Characterization of a Pfemp1 variant from a P. falciparum field is isolate and the development of anti-Pfemp1 antibodies

Thesis

Characterization of a *Pf*emp1 variant from a *P. falciparum* field isolate and the development of anti-*Pf*emp1 antibodies

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ABSTRACT

Rosetting (binding of infected erythrocytes to uninfected ones) is a parasite adhesion phenotype thought to be mediated by *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1). Despite the association between rosetting and severe malaria in Africa, rosette-mediating *Pf*EMP1 variants from African-derived strains are yet to be characterized.

I initially attempted to detect the predominantly expressed *var* genes in recently culture-adapted Kenyan *P. falciparum* isolates selected for rosetting. Unfortunately no clear rosetting-associated genes were detected, possibly because of incomplete selection and lack of a clear phenotype in the selected parasites.

I then went on to study another Kenyan parasite line, SA075, whose predominant expressed *var* gene had interesting sequence features previously linked to rosetting. Using PCR walking, cloning and sequencing, I identified the full-length *var* gene corresponding to the expressed tag sequence, called SA075*var*1. Subsequent experiments failed to confirm a role for the *Pf*EMP1 variant encoded by SA075*var*1 in rosetting. However, SA075*var*1 had domain cassette DC 8-like features which have lately been linked to severe disease.

Antibodies raised in rabbits against recombinant proteins from SA075*var*1 domains revealed functional antibodies with an ability to recognize the surface of parasite infected erythrocytes and to mediate phagocytosis. Although mainly

variant-specific, the antibodies showed limited cross-reactivity with one other parasite strain.

Using plasma pairs from Kenyan malaria patients in acute and convalescent stages of malaria, I examined antibody responses against SA075 and four other rosetting *P.falciparum* strains selected to enrich for single rosette-mediating variants. Antibody responses were seen against particular rosette-selected strains suggesting that some variants may be important for particular severe malaria syndromes.

Overall, the data described here demonstrates the need for continued research into functional and immunological characterization of *Pf*EMP1 variants, especially in field isolates. Antibody data, however, showed clinical relevance of some rosette-mediating variants that could become important targets for malaria intervention studies.

DECLARATION

I declare that the content of this thesis my own work and has not been submitted for any other degree. The process of sample collection and consenting was however done as part of a larger integrated study on the development of natural immunity to malaria in children in Kilifi district.

Carolyne Musoriza Kifude

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Lastly, special thanks to Thathy Vandana and Jose Stoute for allowing us to use the SA075 parasite. I am also deeply indebted to the parents or guardians as well as the children whose samples we have used in this study.

Special thanks to my entire family especially mum and dad for their continued love and support and also my husband Ambrose and lovely daughter Michelle.

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CIDR Cysteine-rich interdomain region

CM Cerebral malaria

CR1 Complement receptor 1

CSA Chondroitin sulfate

DBL Duffy-binding like

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-Linked ImmunoSorbant Assay

FACS Fluorescent activated cell sorting

FITC Fluorescein isothiocyanate

FSC Forward scatter

HEPES 4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid

HI Heat inactivated

IC Impaired consciousness

IFA Immunofluorescent assay

Ig Immunoglobulin

IgG Immunoglobulin G

IgM Immunoglobulin M

LB Luria-Bertani

MFI Median fluorescence intensity

MSP Merozoite surface protein

PAR Palo Alto

PBMC Peripheral blood mononuclear cell

PBS Phosphate buffered saline

PCV Packed cell volume

*Pf*EMP1 *Plasmodium falciparum* erythrocyte membrane protein 1

PCR Polymerase chain reaction

R+ Rosetting

R- Non-rosetting

RBC Red blood cell

RD Respiratory distress

RF Rosette frequency

RIFIN Repetitive interspersed family proteins

RNA Ribonucleic acid

RPMI Roswell Park Memorial Institute

RT-PCR Reverse transcription polymerase chain reaction

SD Standard deviation

SMA Severe malarial anemia

STEVOR Subtelomeric variable open reading frame

SSC Side scatter

TBE Tris/Borate/EDTA

TEV Tobacco Etch Virus

UM Uncomplicated malaria

VSA Variant surface antigens

WHO World Health Organisation

CONTENTS

CHAPTER 1

General Introduction

1.1: *P.falciparum* malaria-a perspective

Although a recent world health organization report (2011) showed an overall reduction in global malaria mortality by 26%, it still continues to be a major public health problem (WHO, 2011). An estimated 216 million cases were reported globally in 2010 with an estimated 655,000 deaths occurring in children under the age of 5 living in sub-Saharan Africa within the same year. In a malaria atlas project study (MAPS), the burden of clinical malaria which is potentially life threatening was shown to be high in certain regions of sub-Saharan Africa and Asia (Figure 1.1) (Hay, Okiro et al. 2010). It is evident from these reports that despite numerous efforts that have led to reduced malaria mortality, more still needs to be done to understand the pathogenesis of severe malaria which often leads to malaria mortality. Definitive understanding of the molecular mechanisms that cause severe malaria is still lacking, hence limiting advances towards development of therapeutics or anti-disease vaccines. This thesis will address an aspect of molecular pathogenesis of severe malaria.

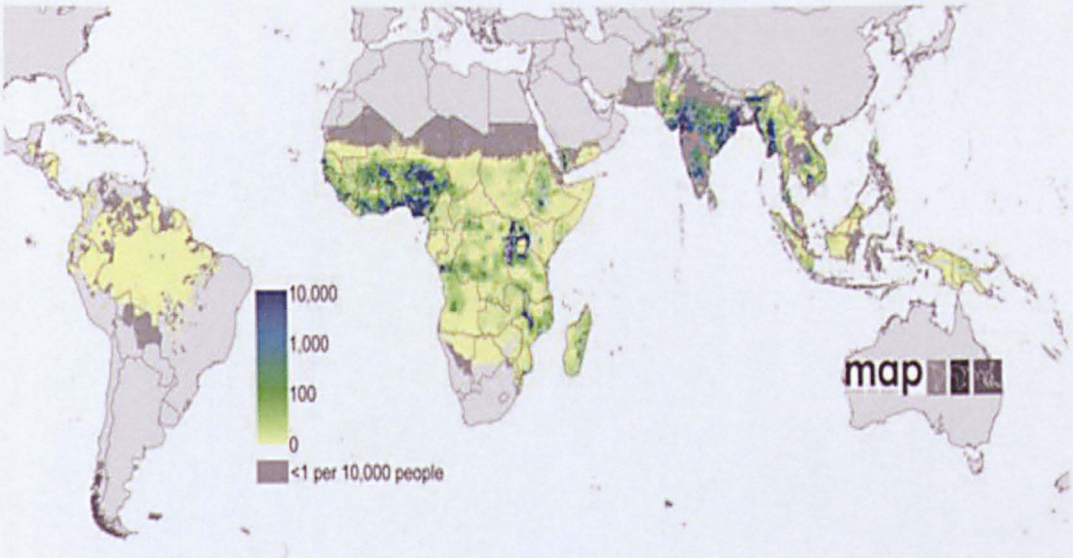


***Figure 1.1**: Estimates of global clinical burden of Plasmodium falciparum malaria in 2007. Figure from Hay et al., 2010 (Hay, Okiro et al. 2010).*

1.2 Severe malaria

Severe malaria is a complicated syndrome whose manifestation is influenced by parasite, host and environmental factors (Figure 1.2) (Miller, Baruch et al. 2002). The overall clinical outcome of malaria infection ranges from asymptomatic infections to severe malaria and death with severe malaria presenting mainly as multi-organ failure, respiratory distress, severe anemia and impaired consciousness (Marsh, Forster et al. 1995; Dondorp, Lee et al. 2008). Severe malaria which is often seen in children in Sub-Saharan Africa may present either as a single syndrome or as overlapping syndromes and at times results in death within 12-24 hours of hospital admission. Another form of severe malaria is the pregnancy-associated malaria (PAM) which causes maternal anemia, low birth weight, premature delivery and increased child mortality (Kane and Taylor-Robinson 2011).

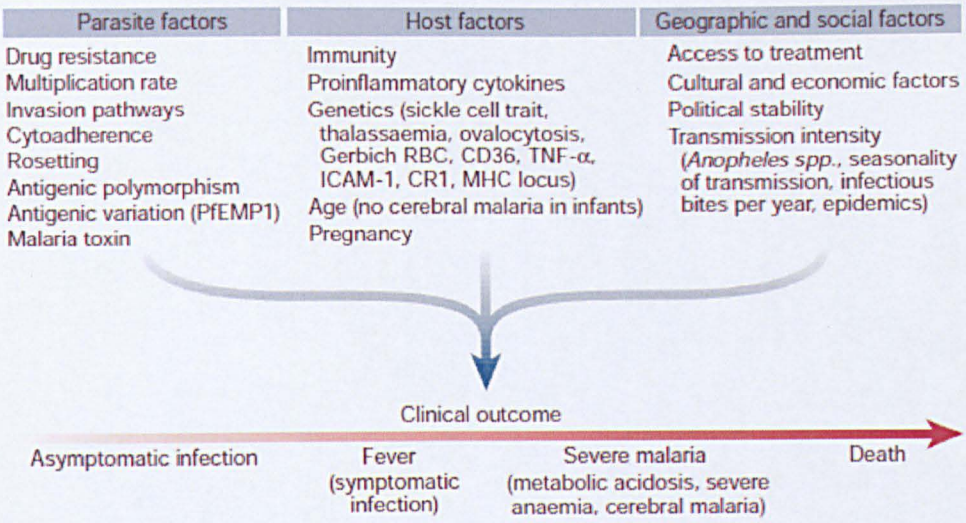


Figure1.2: *Interplay between parasite, host and environmental factors in development of clinical malaria. Also shown is the spectrum of clinical outcome which ranges from asymptomatic infections to severe malaria and death. Figure from Miller L.H et al.2002 (Miller, Baruch et al. 2002).*

In a prospective study carried out by Marsh and colleagues on 1844 children in Kilifi, Kenya (Marsh, Forster et al. 1995), the spectrum of severe malaria was defined and classified into three major syndromes: - impaired consciousness, respiratory distress and severe malarial anemia (Marsh, Forster et al. 1995). Impaired consciousness is a neurological complication whose assessment is based on Blantyre coma Score (BCS). The score is based on motor and verbal response as well as eye movement to determine the extent of the syndrome (Molyneux, Taylor et al. 1989; Waller, Krishna et al. 1995). In the Kilifi study, a BCS score of 4 or less was used as a cut-off for impaired consciousness (Marsh, Forster et al. 1995). Cerebral malaria, which is a form of severe impaired consciousness, is characterized by deep coma and inability to make localizing

response with a BCS of <3 (Molyneux, Taylor et al. 1989; Newton, Chokwe et al. 1997) and no other cause of coma at least 1 hour after termination of a seizure or correction of hypoglycaemia (World Health Organization 2000).

Respiratory distress on the other hand is an important syndrome found to occur in relatively younger children as compared to impaired consciousness (Marsh, Forster et al. 1995). Although traditionally thought to be as a result of cardiac failure (Molyneux 1989; Lackritz, Campbell et al. 1992), respiratory distress is characterized by deep breathing with metabolic acidosis (Marsh, Forster et al. 1995) that may or may not be accompanied by low hemoglobin levels as seen in malarial anemia. The parasite's contribution of acids is often difficult to determine but it is thought to be minor. Thus, fluid replacement and blood transfusion are associated with resolution of acidosis and clinical improvement (English, Sauerwein et al. 1997)

Severe malarial anemia is defined as hemoglobin level of < 5g/dL (Marsh, Forster et al. 1995) and is also thought to contribute to metabolic acidosis by impairing tissue oxygenation. Although rupture of infected erythrocytes by mature parasites is thought to account for the profound anaemia encountered in many malaria patients, other potential causes of may include increased immune haemolysis, phagocytosis (Yuthavong, Bunyaratvej et al. 1990) and splenic clearance (Ho, White et al. 1990) of both infected and uninfected erythrocytes following sensitisation with IgG (Scholander, Carlson et al. 1998) or complement and changes in deformability (Waitumbi, Opollo et al. 2000; Dondorp, Nyanoti et al. 2002).

An overlap of these syndromes was observed in Kilifi and resulted in a mortality rate of up to 34.7% (Marsh, Forster et al. 1995).

There are numerous clinical and epidemiological differences in the presentation of severe malaria syndromes. For example in S.E. Asia, half of the severe *P. falciparum* cases are cerebral malaria (Tran, Day et al. 1996), while in other places, like Papua New Guinea, this number decreases to about 17% (Lalloo, Trevett et al. 1996). Severe malarial anemia has on the other hand been shown to be commoner in areas of high transmission than in areas of low transmission (Snow, Omumbo et al. 1997; Marsh and Snow 1999). Age differences in the manifestation of severe malaria cases are also noted with S.E. Asia reporting more adult cases (Pongponratn, Riganti et al. 1991; Silamut, Phu et al. 1999) while sub-Saharan Africa reports more childhood cases (Taylor, Fu et al. 2004). Even within children in sub-Saharan Africa, clinical manifestations tends to occur at different age-groups with severe malarial anemia occurring in the youngest children while impaired consciousness occurs in slightly older children (Marsh, Forster et al. 1995; Snow, Omumbo et al. 1997; O'Meara, Bejon et al. 2008). A detailed understanding of the molecular basis of severe malaria thus remains crucial. In this thesis, variant specific antibody responses against rosette-mediating variants will be examined to determine whether the variants are linked to any of the clinical syndromes described above.

1.3: What is the molecular basis of severe malaria?

1.3.1: P.falciparum sequestration

P. falciparum sequestration is thought to be the key virulence factor in the pathogenesis of severe malaria. It occurs when mature forms of the parasite disappear from peripheral circulation and adhere onto various host endothelial

cells in different vital organs (Miller 1969; Luse and Miller 1971). It is unknown why parasites sequester but it is thought that they do so to escape from spleen-mediated clearance mechanisms (Mebius and Kraal 2005).

When the *P.falciparum* parasites (in form of sporozoites) get inoculated into the host by the mosquito, they migrate into the bloodstream and invade liver cells where they grow and divide for about 8–10 days (Life cycle shown in Figure 1.3). Daughter cells called merozoites are then released from the liver into the bloodstream where they invade red blood cells (RBCs). The merozoites then go into an erythrocytic cycle where they develop into ring-stage then to trophozoite and schizont-stage parasites within the infected RBCs. The mature schizonts then rupture to release more merozoites which then invade new cells. The entire cycle takes about 48 hours from merozoite invasion to rupture of the infected RBCs, leading to an exponential increase in parasites. It is during the late stages that the parasite modifies the surface of infected RBCs by expressing parasite derived proteins such as *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1). This protein binds to receptors on the surface of vascular endothelial cells, thereby sequestering the infected cells within various tissues. Sequestration is thought to be a crucial factor underlying the pathogenic and virulent nature of *P. falciparum* malaria (Miller, Baruch et al. 2002). One major hypothesis as to how sequestration leads to severe malaria is that the binding of parasites onto the endothelium or to non-infected RBCs (rosettes) may result in occlusion of microvascular blood flow hence impaired oxygen delivery and ultimately dysfunction of affected organs (Kaul, Roth et al. 1991; Dondorp, Ince et al. 2008). This is supported by postmortem studies in which massive sequestration of parasites on the brain endothelium has been observed (MacPherson, Warrell et al. 1985; Pongponratn, Riganti et al. 1991; Taylor, Fu et al. 2004; Ponsford, Medana

et al. 2012), which has been thought to be the underlying cause of coma in cerebral malaria.

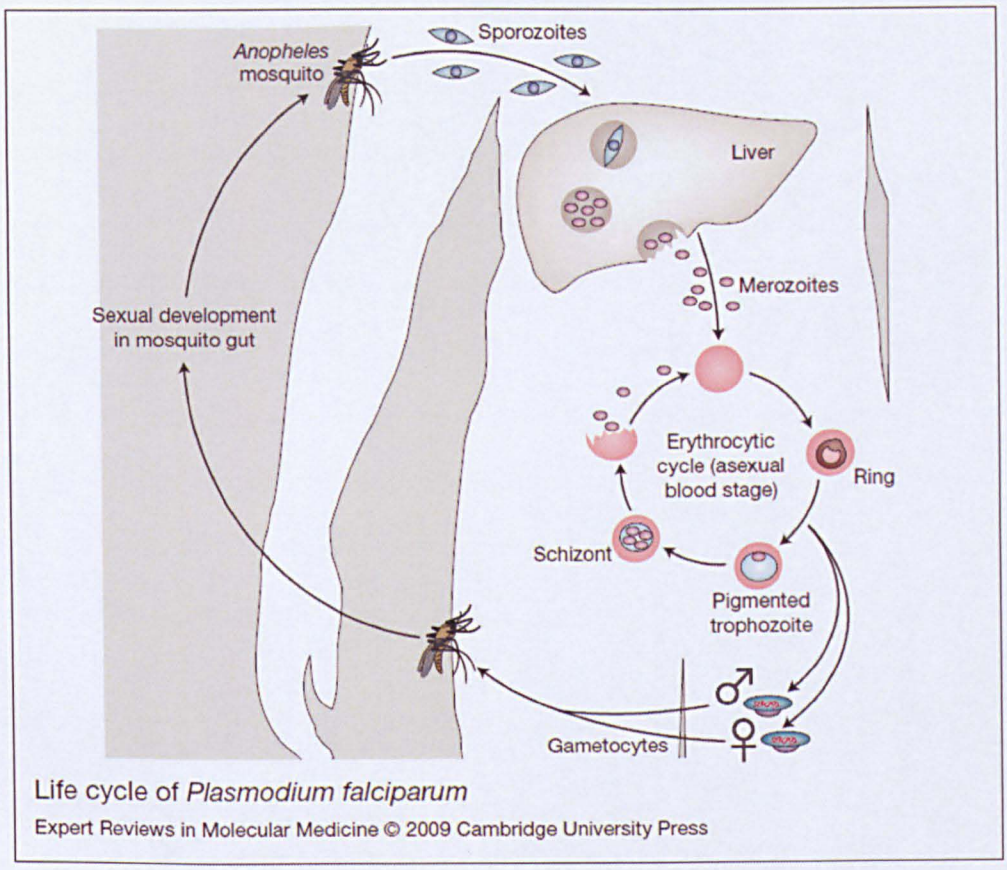


Figure 1.3: *Life cycle of Plasmodium falciparum. Figure from Rowe et al., 2009 (Rowe, Claessens et al. 2009). Details of the life cycle are in the text.*

1.3.2: Host ligands involved in sequestration

Different host receptor molecules have been shown to play a key role mediating sequestration. A review by Rowe *et al.,* 2009 (Rowe, Claessens et al. 2009) highlighted 18 receptors involved in *P. falciparum* cytoadhesion. 12 of these were involved in endothelial adhesion, 3 were involved in rosetting and 3 were involved in platelet-mediated clumping. Other specialised receptors like chondroitin sulphate A (CSA) are involved in binding of infected erythrocytes onto

syncytiotrophoblasts to bring about placental sequestration (Bray and Sinden 1979).

Although not all receptors have been studied for their relationship with severe disease, there are differences in results from association studies done in African and S.E. Asia (Ho, Singh et al. 1991; Newbold, Warn et al. 1997; Heddini, Chen et al. 2001; Heddini, Pettersson et al. 2001). The adhesion molecules involved in rosetting will be discussed later in this chapter.

1.3.3: Parasite ligands involved in sequestration

1.3.3.1 PfEMP1

As mentioned in section 1.3.2 above, the parasite ligand that is thought to play a key role in mediating sequestration is *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Gardner, Pinches et al. 1996). It is encoded by a family of about 50-60 *var* genes in a haploid genome, which are expressed in a mutually exclusive manner (Roberts, Craig et al. 1992; Baruch, Pasloske et al. 1995; Smith, Chitnis et al. 1995; Chen, Fernandez et al. 1998; Gardner, Hall et al. 2002). This controlled antigenic variation of surface-exposed antigenic determinants is a prime immune evasion mechanism used by *P.falciparum* as well as other pathogenic protozoa amd bacterium e.g. African trypanosomes and *Haemophilus influenza*, to maintain a chronic infection in the presence of constant immune pressure exerted by their hosts (Borst and Greaves 1987; Deitsch, Moxon et al. 1997).

Var genes are made up of two exons:- Exon I and Exon II. The extracellular region of *Pf*EMP1 which is encoded by Exon I has an N-terminal segment (NTS) followed by several cysteine-rich domains known as DBL (duffy-binding-like) and CIDR (cysteine-rich interdomain regions) that have been classified into distinct types

based upon sequence similarity (Smith, Subramanian et al. 2000). The number, position and type of DBL and CIDR domains vary among the different *Pf*EMP1 variants in a genome. It is this variable domain composition and extensive sequence polymorphism that is thought to provide great flexibility in sequestration and phenotypic properties (Taylor, Kyes et al. 2000). On the other hand, Exon II which encodes the acidic terminal segment (ATS) is fairly conserved in all *var* genes. The DBLα domain is the most conserved and this feature has been exploited to design "universal primers" that have been used in *var* gene transcription profiling studies (Taylor, Kyes et al. 2000). Figure 1.4 shows an example of domain architecture for a hypothetical *var* gene encoded *Pf*EMP1.

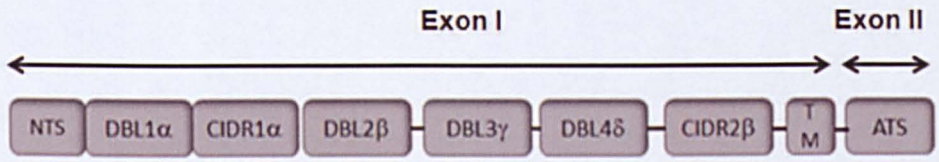


Figure 1.4: *Schematic architecture of a P. falciparum var gene-encoded PfEMP1 protein. The N-terminal extracellular region and the short transmembrane (TM) sequence are encoded by Exon I while the conserved acidic terminal sequence (ATS) is encoded by Exon II. The number and combinations of domains vary considerably between the different PfEMP1 molecules in a genome. NTS: N-terminal segment; DBL: Duffy binding-like; CIDR: cysteine-rich interdomain region; ATS: acidic terminal segment.*

One important aspect of studies on *var* genes has been establishing a link between specific *var* genes with particular adhesion phenotypes and clinical syndromes of severe malaria (Kyriacou, Stone et al. 2006). These attempts have

been made difficult by the extensive intergenomic and intragenomic variation as well as high rates of recombination and mosaicism of *var* genes described in both laboratory and field isolates (Kraemer, Kyes et al. 2007; Bull, Buckee et al. 2008). Nonetheless, different *var* gene classification methods have been adopted and their biological or functional relevance tested in both field and laboratory isolates that exhibit different phenotypes. All the three methods described below will be used in this thesis for classification of *var* genes from field isolates.

1.3.3.2 Classification of var genes

i) Classification based on Upstream (Ups) sequences

This has been the classical method of classification of *var* genes but it is still applicable to date (Smith, Subramanian et al. 2000; Gardner, Hall et al. 2002). Based on sequence homology, upstream sequences (Ups) which are fairly conserved have been classified into 3 major groups: - UpsA, UpsB, UpsC. UpsA *var* genes are found at the sub-telomeric chromosomal locations and are transcribed towards the telomeres. UpsB *var* genes are also mainly located at the sub-telomeric regions but are transcribed away from the telomeres while UpsC are centrally located and are transcribed away from the telomeres (Kraemer and Smith 2006). The clinical relevance of this classification method is evident in field studies in which Ups A *var* and/or Ups B *var* genes are generally transcribed in parasites from children with severe malaria (Kaestli, Cockburn et al. 2006; Rottmann, Lavstsen et al. 2006) while Ups C genes are transcribed in parasites from children with asymptomatic malaria (Kaestli, Cockburn et al. 2006).

ii) Cys/PoLV and block sharing (BS) group classification methods

Due to the highly polymorphic nature of *var* genes, sequence analysis methods based on semi-conserved regions within the DBLα domain have been used to classify *var* genes and to study their relationship with severe disease (Bull, Berriman et al. 2005; Normark, Nilsson et al. 2007). Bull *et al.*, 2005 (Bull, Berriman et al. 2005) classified *var* genes into six groups (Table 1.1) using an algorithm that is based on a 300-400bp semi-conserved region of DBLα, also known as the DBLα "tag" (Bull, Berriman et al. 2005; Bull, Kyes et al. 2007). The main feature of this classification method is the number of cysteine residues within the tag region (hence "Cys") and amino acid motifs occurring at four fixed positions known as positions of limited variability (PoLV) within the tag region. Unique motifs MFK and REY which are mutually exclusively found within the tag sequences have also been incorporated in the algorithm. Cys/PoLV groups 1,2 and 3 have two cysteine residues while groups 4 and 5 have four cysteine residues. Group 6 has either 1, 3, 5 or 6 cysteine residues and is hence referred to Cys X. Also of interest was the observation that cys2 sequences were generally significantly shorter than Cys4 sequences, a feature that could be incorporated in *var* gene sequence analysis. Considering that the high recombination rate between *var* genes which results to extreme diversity, the Cys/PoLV classification method allows for comparison of sequences especially between field isolates (Bull, Berriman et al. 2005; Bull, Buckee et al. 2008; Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012). In this thesis, the six groups referred to as "CP" groups will be used in sequence analysis. Table 1.1 shows the 6 CP groups while the Venn diagram (Figure 1.5) shows how they fit in the larger Cys2 or Cys4 sequence types.

Further analysis has been done on the tag sequences. The string of four amino acids at each of the PoLVs together with the cysteine count forms a sequence signature also known as "sig", which has also been used to further classify DBLα sequences (Bull, Berriman et al. 2005).

Further to this, *var* genes were grouped into seven block sharing groups (BS) in which sequences within a BS group carried exact matches to a collection of 573 14-aa-sequence blocks within highly polymorphic regions of the sequence (Bull, Buckee et al. 2008). At a sensitivity of about 90% and specificity of 100%, 3D7 *var* gene sequences in BS1 group were exclusively found to be of group A type. BS2 on the other hand did not contain any group A sequences but contained about 58% cys2 sequences most of which were from CP2. The biological relevance of these classification methods has been tested in several studies with CP1 being associated with impaired consciousness and CP2 being associated with severe malaria anaemia (Bull, Buckee et al. 2008; Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012). On the other hand, sequences falling in Group A as well as those in CP2/BS1, CP2/BS2 and CP6/BS6 groups have been associated with rosetting (Bull, Buckee et al. 2008). Sequences of sig2 type have also been associated with rosetting (Bull, Berriman et al. 2005; Bull, Kyes et al. 2007).

Table 1.1: *A summary table of classification of var gene sequences into Cys/PoLV groups as described by Bull and colleagues (Bull, Berriman et al. 2005).*

Sequence groups	Number of cysteine residues within the tag region	Unique motif identifying the group
CP1	2	MFK
CP2	2	REY
CP3	2	-
CP4	4	-
CP5	4	REY
CP6	X (1 ,3 5 or 6)	-

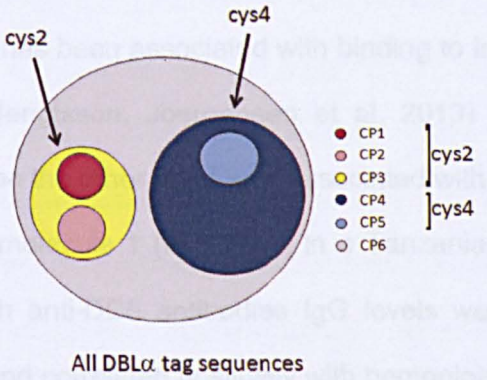


Figure 1.4: *Venn diagram showing DBLα tag sequences by Cys/PoLV (CP) classification method. CP1 and CP2 which contain mutually exclusive MFK and REY motifs respectively are subgroups of Cys 2. CP3 on the other hand is a Cys 2 without any of these 2 motifs. CP4 contains 4 cysteine residues with CP5 being a subgroup that has a REY motif. CP6 contains either 1, 3, 5, 6 or X number of cysteine residues.*

iii) Classification based on domain cassettes -DC

Domain cassette is the latest approach in the classification of *var* genes which is based on the entire gene sequence rather than smaller regions such as the upstream and the DBLα tag region (Rask, Hansen et al. 2010). A domain cassette is defined as two or more consecutive domains belonging to particular subclasses and present in three or more of the 7 *P.falciparum* genomes that were studied. Currently, 24 domain cassettes have been described (Rask, Hansen et al. 2010; Berger, Turner et al. 2013) with two of them showing evidence of association with severe disease. These are DC 8 and DC13 which have both been associated with severe malaria, mainly cerebral malaria and respiratory distress (Lavstsen, Turner et al. 2012; Bertin, Lavstsen et al. 2013). Lately DC8 and 13 have been linked to binding onto endothelial protein C receptor (EPCR) (Turner, Lavstsen et al. 2013), a receptor involved in anticoagulation and endothelial cytoprotective pathways. Although DC4 has been associated with binding to Intracellular adhesion molecule 1 (ICAM1), (Bengtsson, Joergensen et al. 2013) there was no link to severe malaria. DC5 on the other hand was associated with binding to Platelet endothelial cell adhesion molecule 1 (PECAM1) in a Tanzanian study (Berger, Turner et al. 2013) in which anti-DC5 antibodies IgG levels were associated with protection from malaria and correlated positively with hemoglobin levels.

Lately, sequencing and identification of *var* genes has been made easier by the high throughput sequencing techniques (Liu, Li et al. 2012) (Gandhi, Thera et al. 2012). Full-length *var* genes are also being sequenced and assembled thus making studies on *var* transcription easier. Additionally, the *Var*dom 1.0 Server (http://www.cbs.dtu.dk/services/VarDom/), a free software has been constructed for the classification analysis of *Pf*EMP1 sequences. It is based on *var* gene

sequences from 7 parasite genomes (Rask, Hansen et al. 2010) and has improved the study on classification and definition of domain boundaries of *var* genes especially in un-annotated field isolates.

1.4 Rosetting

Rosetting is the spontaneous binding of two or more uninfected erythrocytes to erythrocytes infected with mature asexual parasites (David, Handunnetti et al. 1988) (Figure 1.6). It is one of the most studied adhesion phenotypes of *P.falciparum*. Among the earliest studies to report on rosetting was by Udomsangpetch *et al.*,1989 (Udomsangpetch, Wahlin et al. 1989) where rosetting was seen in both established laboratory isolates as well as fresh isolates from malaria patients. Although mainly described in *P.falciparum*, rosetting occurs in other *Plasmodium* species i.e. *P. vivax, P. ovale and P. malariae* (Udomsanpetch, Thanikkul et al. 1995; Angus, Thanikkul et al. 1996; Lowe, Mosobo et al. 1998) as well as rodent and primate species such as *P.chabaudi* and *P fragile* respectively (David, Handunnetti et al. 1988; Mackinnon, Walker et al. 2002). It is not clear why parasites form rosettes but it is speculated that it could be a mechanism for immune evasion (Ruangjirachuporn, Afzelius et al. 1992) or a mechanism to facilitate quick invasion of merozoites onto the non-infected cells (Clough, Atilola et al. 1998). However, an experiment to test whether rosettes allow quick invasion by protecting against invasion-inhibitory antibodies showed no evidence in support of this (Deans and Rowe 2006).

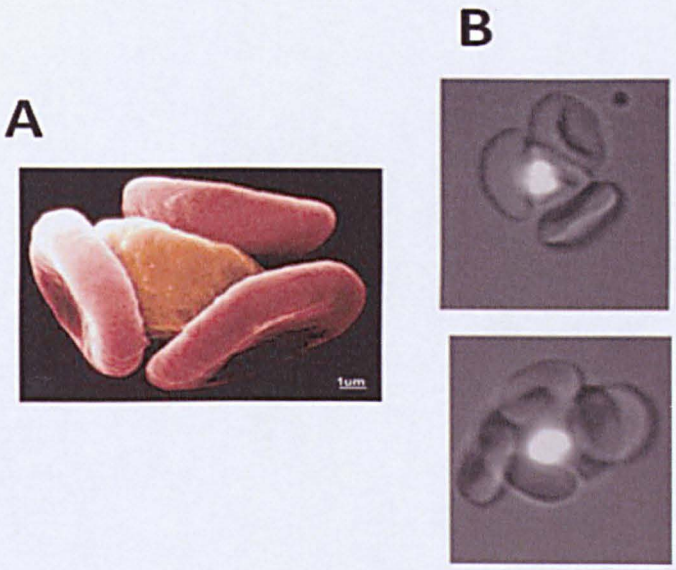


Figure 1.6: A) *Electron micrograph photo showing a rosette in which an infected cell is surrounded by 3 non-infected RBCs. (Image courtesy of David Fergurson, Oxford). B) Routine examination of rosetting parasites under a fluorescent microscope where parasites were stained with 20μg/ml of ethidium bromide and viewed under x40 objective lens. The rosettes shown have 3 and 5 uninfected RBCs bound to an infected RBC.*

1.5 Rosetting and severe malaria

Studies done in sub-saharan Africa from early 1990s to date have consistently shown an association between rosetting and severe disease (Table 1.2). However, this association is not often seen in parts of S.E Asia (Udomsangpetch, Taylor et al. 1996; Angkasekwinai, Looareesuwan et al. 1998). This may be attributed to differences in endemic situations, different parasite population, host genetic makeup, differences in host-parasite interactions and evolution (Mercereau-Puijalon, Guillotte et al. 2008). Table 1.2 is a summary of different studies done in Africa on the association between rosetting and severe malaria (% RF i.e

percentage rosetting frequency as used in the table is a score of the percentage of 200 mature trophozoites that adhered to at least two uninfected erythrocytes).

***Table 1.2**: Rosetting and severe malaria in Africa*

Study site	Study details	Reference
The Gambia		
	24 children with cerebral malaria were compared to 57 children with mild malaria. There was a significant difference in % RF between the two groups (P<0.001)	(Carlson, Helmby et al. 1990)
The Gambia		
	A larger study by the same group as described in (Carlson, Helmby et al. 1990) above. 24 children with cerebral malaria were compared to 106 children with uncomplicated malaria. There was a significant differences in % RF between the two groups (P<0.000001)	(Treutiger, Hedlund et al. 1992)
Madagascar		
	A small study in which three clinical groups were compared. 6 cerebral malaria (CM), 6 severe malaria (SM) and 9 uncomplicated malaria (UM) cases. Both the CM and SM cases had significantly higher % RF than the UM cases. P<0.05 and P<0.002 respectively.	(Ringwald, Peyron et al. 1993)
Kenya		
	Three clinical categories were compared: - 36 severe malaria, 64 moderate malaria and 54 mild malaria cases. There was a significant difference in % RF between severe malaria and mild malaria. P<0.003.No difference was seen between severe malaria and moderate malaria P<0.15. A comparison of cerebral malaria versus all other severe malaria cases showed no difference in %RF. P<0.4.	(Rowe, Obeiro et al. 1995)
Kenya		
	49 severe malaria cases and 45 cerebral malaria cases were compared against 50 children with mild malaria. Severe cases had higher % RF compared to mild malaria. However, a comparison of cerebral malaria against mild malaria showed no difference in %RF.	(Newbold, Warn et al. 1997)

Kenya		
	A study done to compare different adhesion phenotypes between severe and mild malaria cases. Rosetting data showed that 57 severe cases had significantly higher % RF than 64 mild malaria cases. P<0.02.	(Pain, Ferguson et al. 2001)
Kenya		
	A study done to compare different adhesion phenotypes between 21 severe and 45 mild malaria cases. In addition to % RF, size of rosettes and the tightness of rosettes was also evaluated. Severe cases had higher % RF compared to mild malaria cases P<0.001.	(Heddini, Pettersson et al. 2001)
Malawi		
	% RF was determined in fresh isolates from the field. 64 severe malaria cases were compared 32 to cerebral malaria cases (CM), 18 severe malarial anaemia cases (SMA) and 46 CM with or without SMA and uncomplicated malaria. There was no difference in the median % RF for all the groups.	(Rogerson, Tembenu et al. 1999)
Gabon		
	A matched case control study in which 47 severe malaria cases were matched to 47 mild malaria cases. The severe cases had significantly higher % RF in the severe cases than the mild malaria cases P<0.05.	(Kun, Schmidt-Ott et al. 1998)
Mali		
	One of the latest reports on association between rosetting and severe disease. 78 children with severe malaria had significantly higher % RF compared to 91 children with uncomplicated malaria and 40 children with non-severe hyperparasitaemia P<0.0001. No difference was seen in % RF between the different sub-categories of severe malaria	(Doumbo, Thera et al. 2009)

Although rosetting is generally associated with severe disease, the link with specific disease syndromes is uncertain. While most of the studies above showed a clear association between rosetting and severity of clinical malaria (Newbold, Warn et al. 1997; Heddini, Pettersson et al. 2001), only a few of them showed a link between rosetting and specific syndromes such as cerebral malaria (Carlson,

Helmby et al. 1990) (Treutiger, Hedlund et al. 1992) (Ringwald, Peyron et al. 1993). The study by Doumbo *et al.*, 2009 (Doumbo, Thera et al. 2009), however, sought to clarify the association between rosetting and specific disease syndromes. High levels of rosetting were seen in patients with cerebral malaria, severe malarial anemia, non-comatose neurological impairment and repeated seizures without long-lasting neurological impairment. There was no statistically significant difference in the rosetting frequencies between the different syndromes. In Chapter 6 of this thesis, antibody responses against rosette-mediating *Pf*EMP1 variants will help determine whether rosetting is linked to any specific disease syndrome.

Overall, despite these data, there is still limited information as to how exactly rosetting causes severe disease. The positive association between rosetting and severe disease in the studies described above (Table 1.2) does not prove a causal link between the two. It is possible that rosetting may or may not be directly important in pathogenesis or may simply be a marker for some other factors that affect the disease process. However, human genetic studies have shown high prevalence of RBC variants in malaria endemic areas, suggesting their protective role against severe malaria (Hill 1992; Miller 1994). Such polymorphisms that are linked to rosetting include CR1 polymorphisms like Sl(a$^-$) and McC(b$^-$) (Moulds, Kassambara et al. 2000) CR1 deficiency (Cockburn, Mackinnon et al. 2004) and blood group O antigens (Rowe, Handel et al. 2007).

1.6 Molecular mechanisms of rosetting

1.6.1 Which receptors on uninfected RBCs are involved in rosetting?

Different parasites use different host receptors expressed on the uninfected cells to mediate rosetting. As mentioned in section 1.3.2 of this Chapter, three major

receptors on the surface of RBCs have been implicated in rosetting (Rowe, Claessens et al. 2009). These are complement receptor 1 (CR1), heparan sulphate-like molecules and ABO blood group antigens.

CR1 (also CD35) is a receptor on the surface of RBCs thought to be involved in rosetting, with sufficient evidence from a well characterized laboratory isolate (Rowe, Moulds et al. 1997). It is an immune regulatory molecule that binds to activated complement component C3b and C4b to clear away immune complexes (Ahearn and Fearon 1989).

A study by Rowe *et al,* 1997 (Rowe, Moulds et al. 1997) showed that CR1 was the receptor on RBCs that mediates rosetting in R29 parasites. There is also evidence to show the involvement of CR1 in rosetting of field isolates from Kenya and Malawi where rosettes were reversed by an anti-CR1 antibody (J3B11) (Rowe, Rogerson et al. 2000). Using deletion mutants and monoclonal antibodies, this study further showed that the region of CR1 required for the formation of *P. falciparum* rosettes was localized to the area of long homologous repeat regions B and C of the molecule that also acts as the binding site for the activated complement component C3b. Human genetic studies on CR1 polymorphisms provide indirect evidence CR1-mediated rosetting is important in the pathogenesis of severe disease (Moulds, Kassambara et al. 2000; Cockburn, Mackinnon et al. 2004).

Heparan sulphate is a type of glycosaminoglycan (GAG) thought to be involved in rosetting. Although an earlier study on the FCR3S1.2 parasite clone (Chen, Barragan et al. 1998) suggested that heparan-sulphate like molecules were involved in rosetting of this clone, a recent study on the same parasite clarified that

a different *Pf*EMP1 variant may be involved in rosetting whose receptor ligand is not well known (Albrecht, Moll et al. 2011). Nonetheless, field studies done in Cameroon showed that low anticoagulant heparin (LAH), which are related GAGs, had the ability to disrupt rosettes in fresh isolates from children, thus providing evidence that the molecules could be important for rosetting (Leitgeb, Blomqvist et al. 2011).

ABO blood group antigens which are sugars attached to glycoprotein and glycolipids on the surface of RBCs have been shown to play a key role in rosetting (Carlson and Wahlgren 1992; Barragan, Kremsner et al. 2000). ABO blood group antigens have recently been shown to be the main receptor for *VarO* expressing Palo Alto 89F5 rosetting clone (Vigan-Womas, Guillotte et al. 2012). There seemed to be preference for blood group A as opposed to blood groups B and O in the *VarO* expressing parasites. Additionally, the protective effect of blood group O against malaria by mechanism of reduced rosetting (Rowe, Handel et al. 2007) suggests these antigens are involved in rosetting and that they play a role in pathogenesis of severe malaria.

Binding of parasites to non-immune IgM has lately become a phenotype of interest in rosetting of both laboratory and field isolates. Although not directly used as a receptor for rosetting, IgM binding has been shown to be important for rosetting in IT/PAR+ strain where reduced rosetting was seen when IgM was depleted (Clough, Atilola et al. 1998). However, its importance in other IgM-binding rosetting parasites like TM284R+ and HB3R+ is unknown. It is thought that IgM binding acts by either strengthening rosettes (Somner, Black et al. 2000) or by blocking access of variant specific IgG through steric hinderance (Czajkowsky, Salanti et al. 2010). The link between IgM-binding, rosetting and severe disease was first described in

clinical isolates by Rowe *et al.*, 2002 (Rowe, Shafi et al. 2002). More recently, a study by Ghumra *et al.*, 2012 (Ghumra, Semblat et al. 2012) using antibodies to the NTS-DBLα domain of different rosetting isolates showed cross reactivity of the antibodies that seemed to be limited within the parasites with the IgM-binding phenotype. Coupled with the positive association with severe disease in clinical isolates (Rowe, Shafi et al. 2002), IgM-binding in rosetting isolates may be important in the pathogenesis of severe disease and also a good target in intervention studies.

Although the receptors described above have specifically been linked to rosetting, it is reported that there are multiple other possible receptors that could be involved (Chen, Heddini et al. 2000; Heddini, Pettersson et al. 2001), thus highlighting the complexity of this interaction. Complement factors and other serum proteins which are present in the culture media are thought to be important in rosetting (Mercereau-Puijalon, Guillotte et al. 2008). In FCR3S1.2 and Malayan Camp rosetting parasites, complement factor D, serum albumin and natural antibodies to band3 are required for rosetting (Luginbuhl, Nikolic et al. 2007). More studies need to be done especially on clinical isolates to determine the relevant receptors involved in rosetting.

1.6.2: PfEMP1 as rosetting ligand on infected RBCs

Although previous studies had showed that rosettins (Helmby, Cavelier et al. 1993) and rifins (Kyes, Rowe et al. 1999) may be involved in rosetting, there is sufficient evidence to show that *Pf*EMP1 is the key ligand involved in this adhesion. Among the early lab-based evidence to show involvement of *Pf*EMP1 in rosetting is by Rowe *et al* 1997 (Rowe, Moulds et al. 1997). In that study, reverse transcription of the *var* genes showed that R29*var*1 (IT*var*9) was uniquely

expressed in rosetting population of R29 strain and not in the isogenic non-rosetting population. Similar evidence on the role of *Pf*EMP1 in rosetting has been shown for VarO expressing parasites in both primate and human infections (Vigan-Womas, Guillotte et al. 2008). In a different strain called FCR3S1.2, rosetting was recently shown to be mediated by FCR*var*2 (also IT*var*60) (Albrecht, Moll et al. 2011). In addition to R29, Ghumra *et al.,* 2012 (Ghumra, Semblat et al. 2012) also characterised 5 other rosette mediating *Pf*EMP1 variants that were able to induce functional antibodies, including a subset of them that had cross-reactive antibodies. Table 1.3 is a list of *Pf*EMP1 variants that are thought to mediate rosetting in laboratory isolates.

Other than the laboratory-based studies, there is sufficient evidence to suggest that *Pf*EMP1 is involved in rosetting in clinical isolates. Through expression profiling of DBLα tags, data from field isolates suggests that a distinct sub-group of *var* genes is implicated in rosetting in natural parasite populations (Bull, Berriman et al. 2005; Bull, Buckee et al. 2008) and these are also linked to specific disease syndromes (Warimwe, Keane et al. 2009). *Var* gene sequences within Cys 2 or Group A-like were found to be associated with rosetting and severe disease in studies done in Kilifi, Kenya (Bull, Berriman et al. 2005; Bull, Pain et al. 2005; Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012). A different sequence analysis approach by Normark *et al., 2007,* which is also based on motifs within the DBLα tag region, was able to identify three degenerate sequence motifs that correlated with rosetting and severe malaria clinical samples from Uganda (Normark, Nilsson et al. 2007). The mapping of one of the Normark sequences called H3 onto a network of 3D7 and *P.reichenowi* sequences (also known as Kilifi network) described by Bull and others (Bull, Buckee et al. 2008) suggests that it

corresponds to those identified in Kilifi. These are encouraging results and they suggest that there is a limited subset of rosette-mediating variants in natural populations which raises the possibility that a vaccine could be developed specifically against this phenotype.

***Table 1.3**: PfEMP1 variants associated with rosetting in laboratory isolates*

Isolate/Strain/Clone	***Pf*EMP1 variant linked to rosetting**	**References**
TM284R+	TM284*var*1	(Ghumra, Semblat et al. 2012)
HB3R+	HB3*var*6	(Ghumra, Semblat et al. 2012)
PAR+ (also known as FCR3S1.2)	IT*var*60	(Ghumra, Semblat et al. 2012) (Albrecht, Moll et al. 2011)
R29R+	IT*var*9	(Rowe, Moulds et al. 1997)
TM180R+	TM180*var*1	(Ghumra, Semblat et al. 2012)
Muz12R+	Muz12*var*1	(Ghumra, Semblat et al. 2012)
Palo alto 89F5	*Var*O	(Vigan-Womas, Guillotte et al. 2008)
3D7	PF13-003	(Vigan-Womas, Guillotte et al. 2011)

1.7: Rosetting and immunity to malaria

Acquired immunity to malaria develops in individuals following repeated exposure to infection (Langhorne, Ndungu et al. 2008; Doolan, Dobano et al. 2009). Consequently, in endemic areas, manifestation of clinical disease becomes less severe with age as immunity develops (Marsh and Kinyanjui 2006; Doolan, Dobano et al. 2009). Similarly, immunity against variant surface antigen (VSAs) such as *Pf*EMP1 is thought to occur in a piecemeal manner. During the first exposure, individuals develop antibodies that are specific to the infecting VSA variant (Marsh and Howard 1986; Iqbal, Perlmann et al. 1993; Bull, Lowe et al. 1999). Following repeated exposure, a repertoire of variant-specific antibodies develops that can recognize other VSAs expressed by most parasite isolates (Bull, Lowe et al. 1999; Giha, Staalsoe et al. 2000). Evidence for development of variant specific immunity was shown as early as in the 60s using *in vivo* experiments in *P. knowlesi* (Brown and Brown 1965; Brown, Brown et al. 1968). Overall, these observations may suggest that immunity to disease may involve acquisition of antibodies directed against polymorphic target antigens. In this thesis, antibody responses against rosette-mediating variants will be evaluated during (acute) and after (convalescent) clinical disease. Responses generated using the acute plasma will represent antibodies against the current or previous infections carried by the patient while the difference between the acute and convalescent responses will represent variant specific antibody responses.

The protective mechanisms of immunity against rosetting parasites are thought to be in the ability of the antibodies to inhibit rosette formation, disrupt already formed rosettes or to mediate phagocytosis. In laboratory isolates, antibodies generated from recombinant proteins had the ability to inhibit rosette formation or disrupt already formed rosettes (Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al.

2012). The same antibodies were used to opsonize parasites and were able to mediate phagocytosis of mature infected RBCs (Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012). Although the antibody function was mainly strain specific, there was limited cross reactivity that seemed to be restricted within parasites with the IgM binding phenotype (Ghumra, Semblat et al. 2012).

In clinical isolates, the ability of serum to disrupt rosettes in homologous or heterologous parasite has been demonstrated. Carlson *et al.,* 1990 showed how the presence of anti-rosetting antibodies in plasma could have played a role in modifying the outcome of cerebral malaria. In that study which was carried out in The Gambia (Carlson, Helmby et al. 1990), only 2 out of 12 sera (17%) from children with cerebral malaria tested against the patient's own (homologous) parasites *in vitro* exhibited anti-rosetting activity; while as many as 93% (25 out of 27) of the sera from children with uncomplicated disease had the ability to disrupt rosettes in homologous parasites. A different study by Barragan *et al.*, 1998 (Barragan, Kremsner et al. 1998) using plasma samples from Kenya and Gabon, showed an age related build-up of anti-rosetting serum activity against two rosetting strains: -FCR3S1 and TM284 (Barragan, Kremsner et al. 1998). This suggested that the *Pf*EMP1 variants circulating in that population were antigenically similar to FCR3S1 and TM284 and that rosette mediating variants may be limited in a population. More recently, humoral responses against *VarO* rosetting variant were associated with protection against malaria in samples from Benin where severe malaria cases were compared to uncomplicated malaria (Vigan-Womas, Lokossou et al. 2010). Although the protection was independent of rosette disruption in the Benin study, it suggested a possible cytophylic role of the antibodies. Clinical plasma samples have also been tested against recombinant proteins from rosetting variants such as R29 (Mayor, Rovira-Vallbona et al. 2009)

and *VarO* (Vigan-Womas, Lokossou et al. 2010) in ELISA, with results showing sero-prevalence of these antibodies in endemic populations. It is therefore evident that individuals do mount antibody responses specifically against rosette-mediating variants. Whether these variants are of clinical relevance remains to be known.

An interesting observation about immunity to rosetting variants is that while most forms of severe disease decline with age following build-up of naturally acquired immunity, rosetting which is associated with severe disease does not seem to have a clear age profile. There is no clear evidence that rosetting diminishes with age among children admitted to the hospital (Smith, Subramanian et al. 2000; Warimwe, Fegan et al. 2012). However, this may be due to the fact that children admitted to the hospital (such as those we intend to use in the current study) already have relatively high parasitaemia which may bias the samples towards rosette positivity.

1.8 Scope of the thesis

A gap still remains in the study of rosetting parasites that would directly show the importance of rosetting in the pathogenesis of specific clinical syndromes of severe disease. Using plasma from children who have recovered from severe disease, I aimed to evaluate the clinical relevance of rosette mediating variants from well established laboratory isolates and from a putative *Pf*EMP1 variant identified from a field isolate.

Although rosetting is among the most studied adhesion phenotypes of *P.falciparum,* the molecular mechanisms and the exact role of rosetting in severe disease are not well known. This may partly be due to lack of *in vivo* models that

can exactly mimic rosetting and the subsequent development of severe disease in humans. *In vivo* significance of rosette mediating variants therefore needs to be assessed. As discussed in the introduction, the clinical outcome of severe malaria partly depends on parasite and environmental factors. While many *in vitro* studies have been carried out to understand the molecular mechanisms of rosetting, none of them have involved rosetting parasites from sub-Saharan Africa. This is unfortunate because the most consistent association between rosetting and severe disease has been seen in African populations (Table 1.2).

The initial aim of this thesis was to characterize a rosette-mediating *Pf*EMP1 variant from a Kenyan field isolate and to examine the development of rosette-inhibiting antibodies in a Kenyan population exposed to malaria. The hypotheses to be tested were:

1) That there is a limited subset of rosette-mediating PfEMP1 variants in the parasite population, and that individuals living in malaria endemic areas would raise antibodies against the common rosette-mediating variant types.

2) That due to the limited number of rosette-mediating variants in the population, antibodies to rosetting PfEMP1 variants will show cross-reactivity against multiple rosetting isolates.

Chapter 3 of this thesis contains results on preliminary *var* gene transcriptional profiling in rosette-selected culture-adapted clinical isolates from Kilifi. However, no clear rosette-associated *var* genes were identified. This work was done at the KEMRI-Kilifi laboratory.

Chapter 4 then focused on one recently culture-adapted field isolate called SA075 in an attempt to identify, clone and sequence a full-length *Pf*EMP1 variant thought to be involved in rosetting. The SA075 parasite was chosen for this analysis because the DBLα tag sequences had been previously characterized and shown to be of interest (Bull, Buckee et al. 2008). By sequence classification, the dominant SA075 *var* gene transcript belonged to Cys/PoLV group 2, block sharing group 2 and had sequence signature 2 (sig2), all of which have previously been linked to rosetting (Bull, Berriman et al. 2005; Bull, Buckee et al. 2008). Following identification of the full-length gene expressed in SA075 (called SA075*var*1), assays were optimized for expression of recombinant proteins and raising of antibodies from the different domains of the identified *Pf*EMP1 variant. Also presented in this chapter was an interesting finding that SA075*var*1 had DC8-like features based on the recent classification by domain cassettes. DC8 has recently been linked to severe disease (Lavstsen, Turner et al. 2012) and binding to endothelial protein C receptor (EPCR) (Turner, Lavstsen et al. 2013) and therefore further characterization of SA075*var*1 was of interest. This work was done in Alex Rowe's lab, University of Edinburgh.

Chapter 5 focuses on functional and immunological assays used in characterizing rabbit polyclonal antibodies raised against recombinant domains of SA075*var*1. An important assay described in this chapter was the development of a cell sorting protocol using the antibodies generated in Chapter 4. The cell-sorted parasites (frozen as trophozoites) were shipped back to the Kilifi labs and used in surface recognition assays described in Chapter 6.

Finally, Chapter 6 examines the clinical relevance of SA075 parasites and four well-characterised *P. falciparum* rosetting laboratory strains. Plasma samples from malaria patients collected during and after a disease episode were tested against different cell sorted parasites to investigate whether any of these parasite lines are antigenically similar to parasites causing severe malaria in Kenyan children. Further analysis on induced antibodies suggested that despite not being linked to severe disease, some *Pf*EMP1 variants may be more common in natural populations than others.

CHAPTER 2

Materials and methods

This chapter provides detailed methods of techniques that were used in more than one chapter of this thesis. Further additional methods which are specific for each chapter are given within the chapter.

2.1: Study area

The work presented in this thesis was mainly carried out in Kilifi, a district in Kenya that lies along the Indian Ocean coastline (Figure 2.1, left side map). The study area from which the clinical samples were collected lies within the Kilifi Health and Demographic surveillance system (KHDSS) (Figure 2.1, right side map), in which the District Hospital is located. Samples were collected as part of an integrated study on development of natural immunity to malaria in children within Kilifi district (See Appendix 8.1 for the protocol).

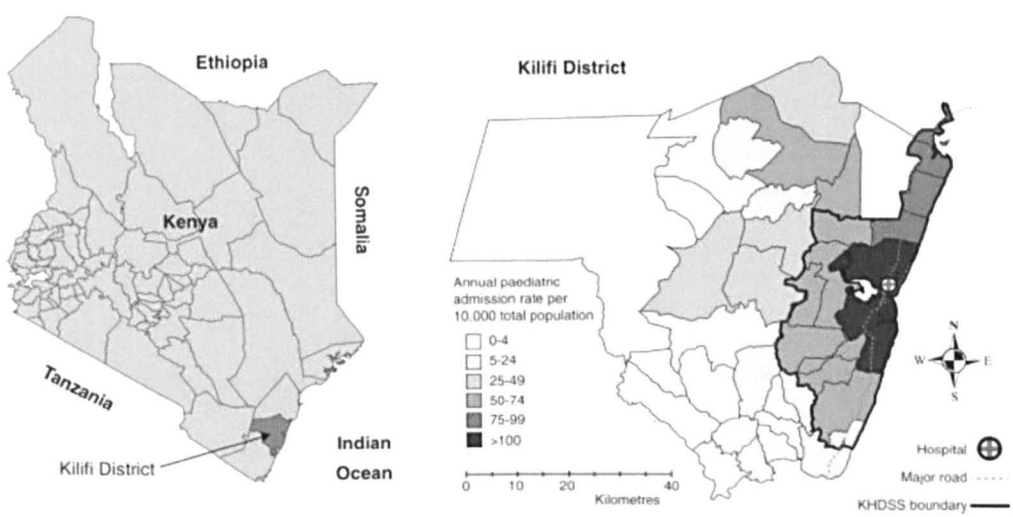


Figure 2.1: *Situation of Kilifi District in Kenya (left) and map of Kilifi District (right) showing the boundary of the KHDSS and the rate of paediatric admissions to Kilifi*

District Hospital by administrative sublocation. Map from Scott et al., 2012 (Scott, Bauni et al. 2012).

Kilifi is a malaria endemic area with transmission increasing after the long rains (April-June) and after the short rains (October-November) each year (O'Meara, Bejon et al. 2008). However, as in many coastal towns in Kenya, malaria in Kilifi has been declining in the last 20 years (Okiro, Hay et al. 2007), with a marked change of pattern in parasite prevalence (O'Meara, Bejon et al. 2008) (Figure 2.2) and a subsequent decline in hospital admissions for malaria (Okiro, Alegana et al. 2009) (Figure 2.3). These changes in malaria epidemiology have been attributed to multiple factors including bed net usage, replacement of chloroquine with sulfadoxine-pyrimethamine as the first line therapy and other social-economic factors (Okiro, Hay et al. 2007). Recent data on the vector population (Mwangangi, Mbogo et al. 2013) also reports a decline in the densities of the major malaria vectors (*Anopheles gambiae* complex) and a shift from human to animal feeding that may have contributed to the decreased burden of malaria along the Kenyan coast.

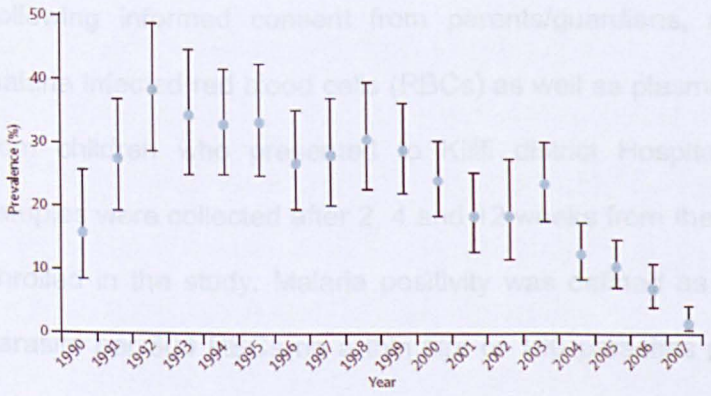


Figure 2.2: *Graph showing a decline in annual parasite prevalence in patients admitted with trauma in Kilifi between 1990 and 2007 (p<0.0001) as described by*

O'meara et al.,2008 (O'Meara, Bejon et al. 2008). The bars represent 95% confidence intervals around the mean.

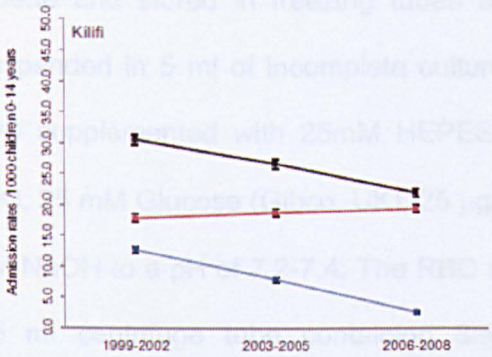


Figure 2.3: *Decline in pediatric admission for malaria (blue line), non-malaria (red line) and all cause-admission (black line) in Kilifi between 1999-2008 expressed per 1,000 children aged 0 to 14 years at risk per annum and 95% confidence intervals as described by Okiro et al.,2009 (Okiro, Alegana et al. 2009).*

2.2: Patient recruitment and sample processing

This study was covered for ethical approval by the Kenya Medical Research Institute (KEMRI) national ethical review board and the Oxford Tropical Research Ethics committee (OXTREC) under protocol SSC no. 1131 (Appendix 8.1). Following informed consent from parents/guardians, acute samples including malaria infected red blood cells (RBCs) as well as plasma samples were collected from children who presented to Kilifi district Hospital. Convalescent plasma samples were collected after 2, 4 and 12 weeks from the same children who were enrolled in the study. Malaria positivity was defined as parasitaemia level of $\geq$1 parasite per 500 RBCs on a thin film or 100 parasites per 100 white blood cells (WBCs) on a thick film.

Upon arrival in the lab, the blood sample, usually about 2-5mls in polypropylene tubes (Falcon®, USA) containing 50 units of heparin (CP Pharmaceuticals Ltd, UK) was spun down to remove plasma. This was then carefully collected using a pipette and stored in freezing tubes at -80°C. The remaining RBCs were re-suspended in 5 ml of incomplete culture media made up of RPMI 1640 (Gibco, UK) supplemented with 25mM HEPES (Gibco, UK), 2mM L-glutamine (Gibco, UK), 25 mM Glucose (Gibco, UK), 25 μg/ml Gentamicin (Gibco, UK), adjusted with 1M NaOH to a pH of 7.2-7.4. The RBC suspension was then gently layered into a 15 ml centrifuge tube containing 3ml Lymphoprep™ (Axis-Shield PoC AS, Norway) to separate out peripheral blood mononuclear cells (PBMCs). This was then followed by centrifugation (Heraeus, Thermo Fisher Scientific Inc.) at 440g for 20 minutes with the brakes off, after which granulocytes were separated by plasmagel flotation. For samples that were malaria positive, the packed RBCs that remained after plasmagel separation were washed twice in incomplete media and divided into 3 portions which were processed differently. Note that the volumes described in i, ii and iii below would vary depending on the original pellet volume obtained from the blood sample.

i. 100μl of ring infected RBCs were frozen down in 1000μl of pre-warmed Trizol® (Invitrogen UK) for RNA extraction.

ii. 100μl of ring infected RBCs was frozen in 1ml aliquots of glycerolyte at -80°C in cryovials (Nunc™, Thermo Fisher Scientific Inc.). The glycerolyte solution contained 42.25 % glycerol, 0.1M sodium lactate, 4mM KCl, NaH_2PO_4, pH 6.8(all from Sigma Aldrich).

iii. Remaining cells were set up in culture as described in section 2.3.1 of this chapter to be frozen down the following day at trophozoite stage.

2.3: Laboratory techniques

The work described in this thesis was done both at the KEMRI labs in Kilifi and at Rowe's lab in University of Edinburgh. There were slight differences in some of the protocols used in the two labs e.g. for rosette selection and preparation of sample for rosette counting. The different methods will be described in section 2.3.4 and 2.3.5.

2.3.1: Parasite culture methods

Parasites were routinely cultured in fresh blood group O RBCs, which were obtained from volunteers in Nairobi. The culture media used to maintain the cultures (herein referred to as complete culture media) was made of RPMI 1640 (Gibco, UK) supplemented with 25mM HEPES (Gibco, UK), 2mM L-glutamine (Gibco, UK), 25 mM Glucose (Gibco, UK), 25 μg/ml Gentamicin (Gibco, UK) and adjusted with 1M NaOH to a pH of 7.2-7.4. This was further supplemented with 10% pooled serum obtained from non-immune donors in Europe. Parasitaemia was maintained up to a maximum of about 10% and media changed daily while consistently maintaining the culture at 2% hematocrit. Culture flasks were incubated in the presence of 3% carbon dioxide, 1% oxygen and 96 % nitrogen (gas mixture was supplied by BOC Kenya).

In order to obtain specific developmental stages of the parasites, synchronization was regularly done on ring stage parasites using 5 % D-Sorbitol (Sigma Aldrich) (Lambros and Vanderberg 1979). To synchronize, the culture was spun down and the supernatant carefully aspirated out. 5 mls of sterile 5% D-Sorbitol solution was added to the cells and incubated for about 15 minutes at 37°C in a water bath. This was then washed twice in incomplete media and re-cultured.

Parasitaemia was determined by Giemsa staining. A 10μl drop of parasite culture resuspended at 40% haematocrit was smeared on a glass slide to make a thin blood film. This was air dried and fixed with methanol (Analar®, VWR International), before being stained with Giemsa (Sigma) diluted to 10% in Giemsa buffer, pH7.2 for 20 minutes. Slides were rinsed with water, air dried for a few minutes and then examined using a 100x objective lens with immersion oil (BDH). Parasitaemia was assessed under a light microscope (Leica Microsystems) by counting 500 RBCs and expressed as the percentage of infected RBCs.

2.3.2: Adaptation of field isolates into in vitro culture

Parasite samples from patients infected with malaria were adapted into *in vitro* culture conditions. Based on criteria established in KEMRI lab, a sample was considered adapted if it stayed in continuous culture for at least 20 cycles to achieve a steady growth and could successfully be frozen down at 5% parasitaemia in at least 10 aliquots that could be re-grown. During the first trophozoite stage *in vitro*, the parasites were tested for rosetting (as described below) and rosetting frequency (% RF) was scored. Field samples took different lengths of time to achieve steady growth in culture, with some taking at least 15 cycles while others taking up to 25 cycles.

2.3.3: Parasites used in this study

Both laboratory and field isolates were used in this study. Field isolates from Kilifi were identified that had different levels of rosetting frequencies for adaptation into culture (Table 2.1). Another rosetting field isolate called SA075, a kind gift from Jose Stoute, was also used. It was obtained from a high transmission area of Kisumu, Kenya. The sample was highly rosetting ex-vivo and was obtained from a patient who had severe malarial anemia. It had been previously characterized for *var* gene expression based on Duffy binding like domain (DBLα) tag analysis (Bull,

Berriman et al. 2005). However, the full-length *Pf*EMP1 responsible for rosetting in SA075 was unknown.

Table 2.1: *Field isolates that were selected for long term adaptation in culture. Also shown are the original rosetting frequencies (% RF) of the samples, their disease syndromes, hemoglobin (Hb) level, Blantyre coma score (BCS) and age of the child at the time of presentation to hospital.*

Sample ID	Rosetting frequency (%)	Disease syndrome	Age (in months)
9197	11	Impaired consciousness, Hb 7.1, BCS 4, No deep breathing	27
8148	23	Non-severe	51
8211	12	Impaired consciousness Hb 10, BCS 2, No deep breathing	34
9106	9	Impaired consciousness and respiratory distress, Hb 5.6, BCS 3, with deep breathing	11
9166	3	Impaired consciousness Hb 8.7 , BCS 3, No deep breathing	46

To examine the clinical relevance of rosetting variants as will be discussed in Chapter 6, five laboratory strains were used in the analysis (Table 2.2). The *Pf*EMP1 variants from the five isolates have previously been characterized in Alex Rowe's lab, University of Edinburgh.

Table 2.2: *Rosetting laboratory strains that will be used in Chapter 6 to examine the clinical relevance of rosetting variants. Also shown are the origin of the parasites, the PfEMP1 variant responsible for rosetting and their IgM binding phenotype.*

Parasite strain	**Origin**	**Rosetting gene**	**IgM binding phenotype**
HB3R+	Honduras	HB3*var*6	IgM positive
TM284R+	Thailand	TM284*var*1	IgM positive
IT/PAR+	South East Asia	IT*var*60	IgM positive
TM180R+	Thailand	TM180*var*1	IgM negative
IT/R29R+	South East Asia	IT*var*9	IgM negative

2.3.4 Rosette selection

Rosette selection for the field isolates started immediately after culture adaptation. Laboratory strains were continuously being selected whenever they were in culture. Selection was routinely done on synchronized trophozoite stage parasites using gelatin flotation (Handunnetti, Gilladoga et al. 1992) and Percoll in alternate cycles every week. Two methods of gelatin-based flotation were used. In the first method which was routinely used in the Kilifi lab, the culture was spun down to remove supernatant and later re-suspended in equal volume of incomplete RPMI in a 15 ml Falcon tube. Pre-warmed 0.75% sterile gelatin made from Porcine Skin, Type A (Sigma Aldrich) was added at an equal volume to the culture suspension. This was followed by a 30-minute incubation in a 37°C water bath until the two layers were clearly visible. By virtue of their density, the rosettes sedimented together with uninfected cells that form rouleaux under these conditions (Handunnetti, Gilladoga et al. 1992). The supernatant containing non-rosetting

trophozoites was obtained, washed with incomplete media and put back into culture as the rosette negative (R-) line while the bottom pellet was maintained as the isogenic rosette positive line (R+). The rosetting frequency was routinely taken for the R+ and R- isogenic lines. This method, however, when done repeatedly could potentially select for knob-negative parasites and eventually make rosette selection difficult. To ensure that the selected R+ lines were also knob positive, rosettes in the R+ culture were periodically disrupted using heparin prior to gelatin selection. This would allow the knob positive parasites to float while the knob negatives would sink to the bottom, hence maintaining knob positive rosetting parasite. To do this, sterile heparin solution (Sigma) was added to 0.75 % gelatin to a final concentration of 3mg/ml. Equal volume of the gelatin/heparin solution was then added to a culture suspension and incubated at 37°C for 30 minutes until there was a clear separation of the two layers. The supernatant containing knob positive-rosetting trophozoites was obtained, washed 3 times incomplete media and put back into culture as before. This procedure was done at least once a month.

The second method of gelatin flotation (a protocol used in the University of Edinburgh Lab) involved gelatin-based solution called Gelofusin (Rhone-Poulenc Rorer). Selection was done at least weekly in alternating cycles with the Percoll method. The culture was first spun down to remove supernatant and later re-suspended in equal volume of incomplete RPMI in a 15 ml Falcon tube. Pre-warmed Gelofusin was added at an equal volume to the culture suspension followed by 10-15 minute incubation in a 37°C water bath until the two layers were clearly visible. The top and bottom layers containing R- and R+ populations respectively were then washed once in incomplete media and once in complete media and re-cultured as described above.

The Percoll method was done at least once or twice a week to enrich rosettes especially in knobless parasites like IT/PAR+(Handunnetti, Gilladoga et al. 1992). A 90 % stock Percoll was first made using 10X RPMI and sterile Percoll (Sigma Aldrich) at a ratio of 1:10 and a pH of approximately 7.0. On the day of selection, further dilutions with 1x incomplete RPMI were done to make a 60 % Percoll solution by adding 5 ml of the 90% Percoll to 2.5 ml of incomplete RPMI in a 15 ml Falcon tube. Parasites were spun down and re-suspended in 5 ml of complete RPMI. This was then gently layered onto the 7.5 ml layer of 60% Percoll and centrifuged at 1500g for 10 minutes with the brakes off. A successful selection had a pellet of uninfected RBCs together with mature-infected rosetting RBCs at the bottom of the tube. A thin layer of non-rosetting mature trophozoites/schizonts was observed at the Percoll/RPMI interface. The supernatant was gently removed by suction and the pellet washed three times in incomplete media before re-culturing.

Samples for RNA extraction were collected during the ring stage prior to rosette selection. About 100 µl of pellet was frozen in Trizol for RNA extraction and subsequent transcript profiling. Stabilates of the parasites were also frozen down in glycerolyte at different points of the selection. Rosetting frequency was recorded at each step of selection.

2.3.5: Assessment of rosetting frequency

Two different methods were used to assess rosetting frequencies. In the first method (done in Kilifi), 0.5 µl of infected RBC pellet at trophozoite stage was re-suspended in 9.5 µl of RPMI containing 5µg/ml Acridine orange (Sigma-Aldrich). 2.5 µl of non-immune AB serum was then added followed by a 30 minutes rotation on a vertical rotator at 24 rpm. 10 µl of the sample was pipetted onto a glass slide,

covered with a 22mmX 22mm cover slip sealed with petroleum jelly for observation under X40 power of fluorescence microscope (Nikon, Tokyo, Japan). Rosetting frequency was scored as a percentage of 200 mature trophozoites that adhered to at least two uninfected erythrocytes. Examples of rosettes are shown in Figure 2.4. Although this method was mainly used to determine the rosetting frequency in thawed out trophozoites, it could equally be used on trophozoites in an ongoing culture.

The alternative method of determining rosetting frequency (done in Edinburgh) involved taking out 200-500 μl aliquot of culture suspension into a 1.5 ml Eppendorf tube. This was then stained with 25 μg/ml of ethidium bromide for about 2-5 minutes at 37°C. Following a gentle mix by flicking the tube, 10 μl of the stained culture was placed on a slide. A cover slip sealed with petroleum jelly was gently lowered and counting done as described above.

Figure 2.4: *Rosetting parasites stained with 5μg/ml Acridine orange as viewed under a fluorescent microscope on x40 objective lens. Shown are infected erythrocytes bound to 3 and 4 uninfected erythrocytes.*

2.3.6: Freezing and thawing of samples

Parasites were frozen down either at ring stage (for re-culturing) or trophozoite stage (to be used once in an assay). Prior to freezing down, a culture at high parasitaemia (usually greater than 5% parasitaemia) was spun down by centrifugation at 400g (Heraeus, Thermo Fisher Scientific Inc.) for 5 minutes in a 50 ml Falcon tube. To 3 volumes of packed cell volume (PCV), 5 volumes of glycerolyte were added in a drop wise manner while gently agitating the tube to ensure even mixing. The first 100µl of Glycerolyte was added and the tube left to stand for 5 minutes before the remaining volume was added in a drop wise manner. 1 ml of the mixture was then transfered into Nunc tubes with proper labelling of the isolate name, parasitaemia, date of freezing and the rosetting frequency. The tubes were then temporarily stored in a -80 freezer before being transferred to liquid nitrogen the following day.

Thawing out of samples was done using three concentrations of sodium chloride (Sigma Aldrich). These were 12%, 1.8% and 0.9%. The latter concentration was supplemented with 20% glucose solution. Frozen parasite samples were removed from liquid nitrogen and allowed to thaw out at room temperature before transferring into a 50 ml tube. 200 µl of the 12% salt was first added in a drop wise manner and left to stand for 5 minutes. This was followed by 10 ml of the second salt (1.8%) and finally 10 ml of the 0.9% salt solution, both in a drop wise manner. The salts were spun down and aspirated followed by two washes with incomplete media. The parasites were then gassed and re-cultured in complete culture media. Frozen trophozoites were thawed out for single use on the day of the assay and were not re-cultured (Kinyanjui, Howard et al. 2004).

2.3.7:Mycoplasma testing

Mycoplasma are small free-living prokaryotes lacking a cell wall and have widely been known to affect 15–80% of all long term cell lines (Rottem and Barile 1993). While *Mycoplasma* does not seem to affect rosetting and cytoadhesion (Rowe, Scragg et al. 1998), it may have an effect on cloning and sequencing of *P.falciparum* genes since its genome is equally AT-rich (Bove 1993; Rowe, Scragg et al. 1998). *Mycoplasma* PCR was therefore routinely done on cultured parasites using a protocol adapted from Tang *et al.*, 2000 (Tang, Hu et al. 2000). The PCR involved a nested technique targeting the 16s and 23s rRNA spacer regions and was able to detect *Mycoplasma M.pirum* as well as acheoplasma *A. laidlawii*, yielding predicted band sizes of between 200-400 base pairs. The PCR was done alongside one commercial control sample of *Mycoplasma*, two positively identified samples of *Mycoplasma* from previously ongoing lab cultures and a no-template negative control. DNA was extracted (Qiagen kit) from 200 µl of culture sample which was spun down and resuspended in 200 µl of 1XPBS. 20 µl of Qiagen protease (or Proteinase K) was pipetted into an Eppendorf tube before the 200 µl sample together with 200 µl of AL buffer and the mixture vortexed for 15 seconds. This was followed by a 10 minute incubation at 56°C after which the tube was centrifuged to remove drops from the inside of the lid. 200 µl of absolute ethanol was added to the sample, mixed and pulse vortexed for 15 seconds. This again was centrifuged to remove drops from the inside of the lid. The mixture was then carefully applied onto a QIAmp Mini spin column followed by a 1 minute centrifugation at 8,000rpm. The spin column was placed in a fresh collection tube and 500 µl of AW1 buffer added and spun down as before. 500 µl of AW2 buffer was then added and spun down at 13,000rpm for 3 minutes after which the empty column was transferred onto the same tube and spun at 13,000rpm for 1 minute to

remove any residual buffer. The column was placed in a clean Eppendorf tube and 200 µl of elution buffer (AE) added followed by a 1 minute incubation and a final spin at 8,000rpm for 1 minute to elute the DNA.

Seven primers, mixed to a final concentration of 1mM were used in two sets (Table 2.3, mix 1 and 2) in a nested PCR. The primers were capable of detecting all eight common *Mycoplasma* contaminants of cell culture. The PCRs were done in a 25 µl reaction containing 1X NH_4 PCR buffer (670mM Tris-HCl (pH 8.8) at 25°C), 160mM $(NH_4)_2SO_4$, 0.1% stabilizer), 1.5mM $MgCl_2$, 1mM dNTP mix, 20µM of either primer mix 1 or 2, 5U/µl of Taq polymerase (all from Bioline Biotaq polymerase kit, UK), 19µl of DNase free water and 1 µl of DNA. Reactions for both PCRs were done at similar conditions i.e. initial denaturation of 94°C for 3 minutes, followed by a 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. 10 µl of PCR products were run on a 1% agarose gel at 90V for 1 hour (described in section 2.3.9.4). Staining was done using 0.5µg/ml ethidium bromide solution and de-stained using distilled water. Visualization was done using Bio-rad Geldoc.

Table 2.3: *Primers used in Mycoplasma PCR. Mix 1 was used for the first step while Mix 2 was used in the second step PCR.*

	Mix 1
1	ACACCATGGGAG(C/T)TGGTAAT
2	CTTC(A/T)TCGACTT(C/T)CAGACCCAAGGCAT
3	AAAGTGGGCAATACCCAACGC ATCC
4	TCACGCTTAGATGCTTTCAGCG ATCC
	Mix 2
5	GTG(C/G)GG(A/C)TGGATCACCTCCT
6	GCATCCACCA(A/T)A(A/T)AC(C/T)CTT
7	CCACTGTGTGCCCTTTGTTCCT

2.3.8: Parasite genotyping using Merozoite surface protein 1 and 2 (MSP1/MSP2) markers

When carrying out studies such as this one, it is imperative to associate specific phenotype characteristics of the parasite with specific genotype /expression profiles. However, previous studies have shown that *in vitro* adaptation of malaria parasites into culture often resulted to loss of strains or clones (Nsobya, Kiggundu et al. 2008; Cheeseman, Gomez-Escobar et al. 2009). Parasite genotyping was therefore done on both acute and rosette selected samples using MSP1/MSP2 PCR that targets the polymorphic merozoite surface protein gene to detect three different allelic variants: -A) MSP2 (IC allele) B) MSP2 (FC allele) and C) MSP1 (Mad20 allele). This was done to check whether the parasite genotypes at acute stage were similar to the genotypes in the rosette selected lines. It was also important to ensure that the R+ and R- lines were isogenic and that they were the same as the original parasite.

A PCR method described by Snounou *et al.*, 1999 (Snounou, Zhu et al. 1999) was adapted but with modifications:- only the allelic family specific primers were used and not the outer primers that flank the conserved regions of the genes. Sequences for the allele specific primers that flank the polymorphic region were as follows: -Mad 20 Forward 5'-AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC -3', Mad 20 Reverse 5'- GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA -3', FC Forward 5'- AAT ACT AAG AGT GTA GGT GCA RAT GCT CCA- 3', FC Reverse 5' -TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC-3', IC Forward 5'-AGA AGT ATG GCA GAA AGT AAK CCT YCT ACT 3' IC Reverse 5'-GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG-3'. The 25 µl PCR reaction was carried out in 1x PCR buffer II (100 mM Tris-HCl, 1.5mM NaCl pH 8.3, 500 mM KCl), 2mM dNTPs, 25 mM $MgCl_2$, 10 µM forward and reverse primers, 5U/µl of Taq polymerase (AmpliTaq® gold) and 100ng of DNA (All from Applied Biosystems). The amplification conditions were:-Initial denaturation of 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, annealing at *45°C for 30 seconds and 72°C for 2 minutes. A final extension was then done at 72°C for 10 minutes. The annealing temperature represented by the asterix mark differed for MSP1 and MSP2 alleles (58 °C and 45°C respectively). PCR products were run on 2 % agarose gels (described in section 2.3.9.4) using 0.5X TBE buffer for one and half hours at 90V. The gel was stained using 0.5µg/ml ethidium bromide solution and later de-stained using distilled water. Visualization was done using Bio-rad Geldoc.

2.3.9: Var gene expression profiling

This section describes a series of steps leading to profiling of *var* gene transcripts in field isolates. They include RNA extraction, generation of cDNA, amplification of *var* gene transcripts using universal primers, sequencing of the DBLα-tag,

classification of sequences and finally identification of the dominantly expressed *var* gene.

2.3.9.1: RNA extraction

Trizol samples from section 2.2 were obtained from storage at -80°C and allowed to thaw for 3-5 minutes at room temperature in the fume hood. Once thawed out, the samples were transferred into a 1.5 ml RNase free screw-cap tube (Starlab, Germany). If the volume of Trizol was less than 1100µl, it was topped up using pre-warmed Trizol in a fume hood. Using P1000 filter tips, 200µl of chloroform (Sigma Aldrich) was added and mixed vigorously for 15 seconds before allowing it to stand for 2-3 minutes. This was followed by spinning at 1400g on a microfuge (Heraeus Biofuge pico) for 35 minutes at 4°C.

While the sample was spinning, fresh RNA tubes were labelled and 2µl Glycoblue ™ (Ambion, UK) added into each of them to allow for subsequent visualization of the pelleted precipitate. The spun samples were gently removed from the centrifuge and allowed to rest for 2 minutes since this tended to increase the yield of the aqueous phase. The clear aqueous phase was then carefully harvested using a pipette into the Glycoblue-containing RNA tubes and 500µl isopropanol (IPA) (Sigma-Aldrich) added. For those with less than 500µl aqueous layer, an equivalent volume of IPA was added. This was then mixed thoroughly by inverting at least 10 times before incubating overnight at 4°C to allow for RNA precipitation. The remaining thin aqueous layer and Trizol pellet was kept in the fridge for harvesting lower quality DNA.

The following day, samples were spun on the microfuge at 16060g for 30 minutes at room temperature and the pellet gently washed with 500µl ice-cold 75% ethanol

by inverting and pouring off the supernatant. During this time, care was taken so that the blue pellet was not washed off in the process. A short spin (2 seconds) was done to bring down the pellet and to completely aspirate off the remaining ethanol using a P20 pipette and filter tip.

The tubes were then inverted on tissue paper and allowed to dry for no longer than 5 minutes. 20μl RNA secure (Invitrogen, UK), a non-enzymatic reagent that irreversibly inactivate RNases, was added into all tubes using a fresh tip each time before heating the sample at 60°C for 10 minutes. The RNA suspension was gently mixed by pipetting up and down using a P20 pipette then finally spun on the microfuge at 380g for 10 seconds to ensure that the 20μl volume was at the bottom of the tube and not on the sides. Finally the RNA was stored at –80°C awaiting reverse transcription PCR (RT-PCR).

2.3.9.2: cDNA preparation

cDNA preparation was done in a PCR hood in room that was free from contaminating PCR products. 2μl of RNA (about 1μg) was transferred into 500μl RNAse treated tubes (Neptune) for cDNA synthesis. The process started by DNA digestion, in which 1μl of enzyme DNase (Ambion Inc., UK) was added to the RNA sample in the presence of 1X DNase buffer (100 mM Tris, pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$) (Ambion Inc., UK) diluted in Ambion DEPC water and incubated for 20 minutes at 37°C. To inactivate the enzyme, 3μl of DNase inactivation slurry (Ambion Inc., UK) was added, mixed and incubated for 2 minutes at room temperature. This was then spun down on the microfuge for 1.5 minutes at 9500g to pellet down the inactivation slurry.

8 µl of the DNase digested preparation was then carefully transferred into PCR strip tubes. For each sample, two aliquots were made which were to be processed with and without reverse transcriptase enzyme (RT+ and RT-). All reagents for this step were from Invitrogen UK. 50ng/ µl of random hexamers and 10mM dNTPs were first added into each of the tubes and placed on a thermocycler for 5 minutes at 65°C followed by 1 minute at 4°C. The reverse transcription reaction was then done using 1X first strand buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$), 25mM $MgCl_2$,0.1 M Dithiothreitol (DTT) and 1µl of RNase inhibitor i.e. RNAseOUT. In every RT+ tube, 1 µl of Superscript II ™ enzyme was added. For the RT- control reaction, 1 µl of DEPC-treated water was added. The tubes were then incubated under the following conditions on a thermocycler :- 25 °C for 10minutes, 42 °C for 50 minutes, 70 °C for 15 minutes and a final 4 °C for 1 minute.

Following the incubation, 0.25 µl of 2U/µl RNase H (Invitrogen UK) was added and incubated at 37 °C for 20 minutes. The role of RNase H was to digest any remaining RNA template after the first strand cDNA synthesis. Finally, two aliquots of 10 µl volumes were made for each sample and stored at -20 °C, with one being for back up. 1µl of the cDNA was used for PCR amplification of DBLα sequence tags as described in the section 2.3.9.3. For genomic DNA, 1µl of the DNAse1™untreated RNA sample was used at a dilution of 1:10
(diluted in 1X Tris EDTA, Sigma-Aldrich).

2.3.9.3: Cloning and sequencing of DBL alpha tag

The amplification of DBLα "tag" as described in this chapter refers to a 300-400bp region of the DBLα domain of the *Pf*EMP1 molecule (Figure 2.5). It has consistently been used to characterize the *Pf*EMP1 molecule since it contains a

semi-conserved region that can be successfully amplified in most of the 60 *var* genes in a genome. The DBLα tag sequences were generated as previously described by Bull *et al.,* 2005 (Bull, Berriman et al. 2005). Briefly, cDNA was amplified using primers DBLαAF 5' GCACG (A/C) AGTTT(C*/T) GC3' and DBLαBR 5' GCCCATTC (G/C) TCGAACCA 3' that were a modified from the originally described AF and BR primers (Taylor, Kyes et al. 2000). The nucleotide marked with an asterisk on the DBLαAF primer indicates the modification that was introduced to broaden the range of sequences that could be amplified. In each of the cDNA amplifications, negative controls (without reverse transcriptase treatment) were included to ensure that the cDNA was not contaminated with gDNA. A parallel gDNA from the each of the sample was also amplified and the *var* gene profiles analyzed to check for any primer bias (Taylor, Kyes et al. 2000).

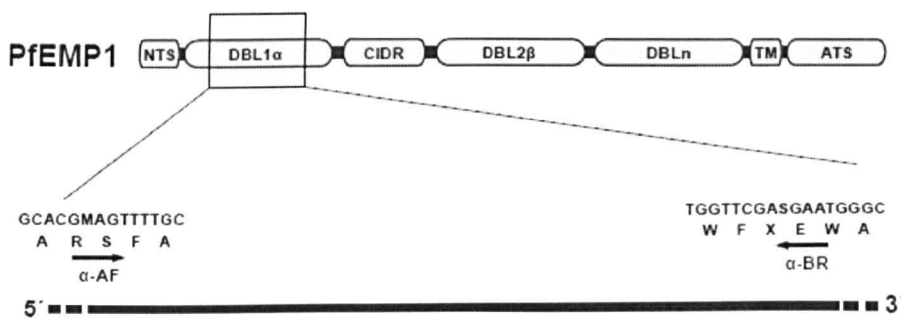


Figure 2.5: *Schematic representation of a PfEMP1 molecule showing the semi-conserved tag region (marked in a rectangle) within the DBL1α domain. The tag region which is approximately 300-400bp was amplified using the DBLαAF and DBLαBR primers as shown.*

Following successful amplification of the DBLα tags, the products were purified using Sephacryl S-400™ columns (Amersham Biosciences, UK). 700μl of the

Sephacryl S-400 was added into empty microspin columns and centrifuged at 380g for 1 minute. The collection tube was emptied after which two further washes were done on the Sephacryl using 200µl of 1X TE buffer. The microspin column was then transferred into clean collection tube after which the 20µl PCR product was added. A short 2 minute incubation was done followed by a 2 minute spin at 380g. The purified product was collected and frozen down in -20°C.

2µl of the cleaned PCR product was then ligated into pCR®2.1-TOPO® vector (Invitrogen, UK) by mixing it with 1µl of salt solution *(*1.2M NaCl, 0.06M $MgCl_2$*)*, 2.5µl of water and 0.5µl of the vector. The mixture was incubated for 5 minutes at room temperature to allow for ligation. Transformation of bacterial cells was done by taking 2µl of the reaction mix and gently mixing with 25µl of One Shot® TOP 10 Chemically Competent E. coli (Invitrogen, UK*)* followed by a 30 minute incubation in ice. A heat shock reaction was done at 42°C for 30 seconds in a water bath immediately followed by 2 minute incubation in ice. The transformed cells were then re-suspended in 500 µl SOC medium (Invitrogen, UK) and incubated for 1 hour in a shaking incubator (200 rpm) at 37°C. Meanwhile, pre-made Luria Bertani (LB-Ampicillin) agar plates were warmed at 37°C. The plates were made by adding 35g of LB agar powder (Sigma) into one litre of water followed by autoclaving. On cooling down, Ampicillin was added to a final concentration of 50µg/ml and the solution poured out onto 100mm petri dishes. 50µl of the bacteria was plated together with 40µl of 20 µg/ml of 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-Gal) for blue/white screening of colonies and 40 µl of 20µM isopropylthiogalactoside (IPTG) which induces the expression of the protein followed by an overnight incubation at 37°C in the presence of 5% CO_2.

Successfully transformed cells formed white colonies while poorly transformed or those in which transformation was unsuccessful formed blue colonies. 16 white colonies were then picked and placed in 3 ml of LB broth in a 15 ml Falcon tube, followed by another overnight incubation on a shaking incubator (200rpm) at 37°C. The following day, plasmid DNA was extracted from the bacterial cells using a miniprep kit QIAprep Spin miniprep kit (Qiagen) in which 1 ml of bacterial cultures from individual colonies was first centrifuged at 13000 rpm for 3 minutes. The pellet was then resuspended in 250 µl buffer P1 (6.06g/l Tris base, 3.72 g/l EDTA, pH 8.0) before lysis with 250 µl of P2 buffer (8g/l NaOH, 1% (w/v) SDS in water) and subsequent neutralization with 350 µl N3 buffer (confidential composition). Cell debris were removed by centrifugation at 13000rpm for 10 minutes before transferring the suspension to a spin column. This was spun down at 8,000rpm for 1 minute followed by centrifugation of an empty spin column to remove residual reagents. Elution buffer was added followed by a final elution of the plasmid DNA. A big dye reaction was done on the DNA and it consisted of 5 µl of the plasmid, 4 µl of big dye and 1 µl of M13 forward primer 5'-GTAAAACGACGGCCAG-3. The reaction mix was incubated for 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes followed by sequencing on an Applied Biosystems 3730 sequencer.

2.3.9.4 Agarose gel electrophoresis

All PCR products were run on approximately 1-2 % agarose gels which were prepared using 1-2 g electrophoresis grade agarose (Invitrogen) in 100mls of 1X TBE (1M Tris, 0.9M boric acid and 0.01 M EDTA, Invitrogen). This mixture was warmed in a microwave oven until all agarose dissolved. The liquid was then careful poured in a cast and left to cool down to solidify. PCR products were then

loaded with 1 μl of dye buffer (60mM Tris-HCl, pH 7.5, 60mM EDTA, pH 8.0, 0.36% Xylene Cyanol FF, 2.4% Orange G, 30% glycerol. DNA ladder was also loaded alongside the products. Gels were run at 80V-100V for 45-60 minutes after which they were removed and stained with 0.5 μg/ml ethidium bromide for 5 minutes before destaining for a further 5 minutes. DNA products were visualized under UV.

2.3.9.5: DBLα tag sequence analysis

DBLα sequence tags from each parasite isolate were classified based on cys/PoLV, a sequence homology grouping algorithm previously described by Bull and colleagues (Bull, Berriman et al. 2005). The raw sequences were run on a Perl script called "raw reads sequence organizer 4" written by Pete Bull which performs DNA orientation, trimming off both the vector and primer sequences before translating them to amino acids. Due to the small number of sequences, translated sequences were aligned using BioEdit sequence editor and checked manually for the presence of a consensus motif DIGDI within homology block D and a consensus motif PQYLR within homology block H at the start and end of the tag sequence respectively. The sequences were then classified into one of six sequence groups based on a count of the number of cysteine residues within the tag region and a set of sequence motifs at four positions of limited variability (PoLV1–4) (Bull, Berriman et al. 2005). Unique sequence herein referred to as sequence "types" were counted for each isolate and expressed as a proportion of the total number of sequences obtained from that isolate.

2.3.10: Flow cytometry and immunofluorescence assays for recognition of PfEMP1 on live infected erythrocytes

Fluorescent Activated Cell-Sorter (FACS) and Immunofluorescence assay (IFA) were done to investigate antibody responses to the native *Pf*EMP1 expressed on

the surface of mature trophozoites. The antibodies used for these assays were polyclonal, targeting different domains of rosette-mediating *Pf*EMP1, and were raised in rabbits. Rabbit pre-immune and immune anti-serum was also used. The assays were done for the following reasons: -

i) To screen the pre-immune sera prior to immunization of rabbits. This was due to the presence of heterophile antibodies in some rabbit sera that would otherwise react with human RBCs (Rowe, Shafi et al. 2002).

ii) To determine which rabbit anti-sera with the greatest signal was to be selected for IgG purification.

iii) To test for surface recognition of homologous parasites using rabbit anti-*Pf*EMP1 IgG to the different domains of the rosette mediating *Pf*EMP1.

iv) To test for cross reactivity of the purified IgGs, i.e. anti-NTS-DBLα antibodies of the rosette-mediating *Pf*EMP1 variant across a panel of different *P.falciparum* strains.

v) To work out the proportion of *Pf*EMP1 positive cells in rosetting parasites before and after cell sorting and to compare them with % RF.

From the same material used in the FACS assay, a thin smear was made and used for the IFA microscopy. A working protocol for the combined FACS/IFA assay is in appendix 8.2. A different FACS protocol (described in Chapter 6) was used specifically for the assays involving plasma from clinical samples.

Briefly, 1.2 ml of culture suspension (mature pigmented trophozoites) at 2% haematocrit was aliquoted into an Eppendorf tube. 2μl of Hoechst 33342 dye (Invitrogen molecular probes) used to stain parasite DNA was then added to a final concentration of 1.25 μg/ml followed by 20 minute incubation at 37°C. This was

then washed twice in 750μl of 1X PBS and re-suspended in 1.2 ml of 1% Ig-free BSA in I XPBS also containing 1.25μg/ml Hoechst and 50μg/ml Fucoidan (Sigma). The role of the Fucoidan was to keep the rosettes disrupted throughout the staining and acquisition process.

Apart from assays that were to determine the effectiveness of the antibodies by serial dilution, the antibody concentration used for surface recognition was optimized to 400μg/ml. From a 7.5mg/ml working stock, 5.4 μl of antibody was added to 94.6 μl of parasite suspension and incubated on ice for 1 hour, flicking the tubes every 10 minutes to keep the cells in suspension. This was followed by three washes using 750 μl of 1X PBS. The secondary antibody, Alexa Fluor 488-labeled goat anti-rabbit IgG (Catalogue number A11034, Invitrogen Ltd, Paisley, UK) was diluted 1:1000 and 100 μl added to each tube. This was also followed by a 1 hour, incubation on ice while flicking the tubes every 10 minutes. After another three washes in 750 μl of cold 1X PBS containing 50μg/ml Fucoidan the sample was split into two tubes labeled 1-11 (for IFA) and 1*-11* (for FACS).

For IFA, the supernatant was removed from the final wash and the pellet re-suspended to 20-30% haematocrit in 1XPBS/1% Ig-free BSA followed by thin smears which were air dried and a drop of mountant added i.e.1.25 mg/ml of diazabicyclo-[2,2,2]octane (DAPCO) in 50% glycerol/50% PBS. These were then covered with 22mm X 22mm cover slips and sealed at the edges with a nail polish before storing at 4°C. IFA slides were viewed under X 100 magnification with oil using a Leica DM LB2 fluorescence microscope and images taken with a Leica DFC300FX digital camera. The percentage of *Pf*EMP1-positive cells was determined by counting them against 100 positive infected RBCs per slide. All

adjustments to the photos were applied equally to *Pf*EMP1 antibody and control images.

For FACS assays, the supernatant was removed from the final wash and the pellet re-suspended in 200µl of 0.5 % paraformaldehyde (Sigma-Aldrich) followed by a 20 minute incubation for fixation on ice. This was then spun down to remove the supernatant followed by one wash with FACS buffer containing 1XPBS, 0.5% BSA and 0.01% sodium azide. The final pellet was re-suspended in 500µl of the FACS buffer and transferred into a FACS tube. 25µl of 1mg/ml Fucoidan was added into each tube and incubated for 20 min at room temperature. Tubes were wrapped in foil paper and kept in the fridge until the FACS run (preferably within two days of staining).

Acquisition was done on a Becton-Dickinson LSRII Flow cytometer in which 500,000 events per sample were analyzed. Flow cytometer settings were first adjusted to achieve a clear separation of uninfected (Hoechst-negative) and infected (Hoechst-positive) erythrocytes in a Forward scatter (FSC)/ Hoechst dot plot. Finally data was acquired on FL1 (Alexa 488) and FL2 (Hoechst). The data files usually in FCS format, were exported and analyzed using FlowJo software (Tree Star Inc.). The Alexa 488 fluorescence was used to quantify antibody recognition of *Pf*EMP1. This was expressed either as the mean or median fluorescent intensity or as percentage positive cells.

2.3.11: Mamalian cell culture_THP-1 cells

THP-1 cells (ATCC®TIB-202™) were used for antibody mediated phagocytosis assays. These are monocytic cell lines originally derived from a one year old boy with leukemia and which express complement (C3) Fc receptors (Tsuchiya,

Yamabe et al. 1980). Routine maintenance of the cells was done as described by Ataide *et al.*, 2011 (Ataide, Mwapasa et al. 2011) in which cells were maintained in RPMI 1640 media (GIBCO) supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS) (GIBCO), 1% penicillin-streptomycin (GIBCO), 2 mM L-Glutamine (GIBCO), 25mM HEPES (GIBCO) and 5.5 μM 2-mercapto-ethanol (Sigma). Cells were grown to a concentration of not more than $1X10^6$ cells/ml and the media was changed every 2 to 3 days. As a recommendation by the manufacturer, THP-1 cells were used up to around 5 passages to avoid a possible change in properties.

To thaw out THP-1 cells, the vial containing the cells was warmed in a water bath at 37°C. 5 mls of warm culture media was then added into a 15 ml Falcon tube and THP-1 cells were gently pipetted into the media. This was then spun for 5 minutes at 300g. The supernatant was gently poured out and the cells were transferred into a flask containing 10 mls of warm THP-1 media. 10 μl of 2-β mercaptoethanol (Stock was at 1000X) (Sigma-Aldrich) was added and the flask placed into a 37°C CO_2 humified incubator in an upright position.

Freezing of THP-1 cells was done when the density was about $1x10^6$ cells in 20 mls of media. The cells were centrifuged at 1000g and gently re-suspended in 5 mls of Foetal Calf Serum (FCS) supplemented with 10% DMSO as a cryoprotectant. This was then stored in -80°C for not longer than 24 hours before transferring to liquid nitrogen.

2.3.12 Phagocytosis assays

The protocol used in phagocytosis assays was adopted from Ataide *et al.*, 2010 (Ataide, Hasang et al. 2010) and later slightly modified as described in (Ghumra,

Semblat et al. 2012). Undifferentiated THP-1 cells (ATCC®TIB-202™) were used. On the day of the assay, parasites at trophozoite stage were spun down and re-suspended in RPMI containing 2% heat inactivated FCS with 200 μg/ml Fucoidan. This was run down through a pre-equilibrated magnetic-activated cell separation (MACS) column and the stuck parasites eluted with the RPMI media. A drop of the eluted parasites was used to make a thin smear which was giemsa-stained and viewed under a microscope to ensure that the purification was successful before a parasite count was done with the heamocytometer and diluted down to the optimized parasitaemia of 3.3 $X10^7$ parasites/ml. Ethidium bromide was then added to the parasites to a concentration of 10μg/ml and the mixture incubated for 15 minutes. This was followed by 3 washes and the pellet re-suspended back to 3.3 $X10^7$ parasites /ml. 30 μl of parasite suspension was then opsonized with 3.3 μl of antibodies that were serially diluted to give concentrations ranging from 100μg/ml down to 6.25μg/ml in a 96 well-U bottomed plate pre-coated with heat inactivated FBS. The plate was then incubated for 1 hour at room temperature in the dark to allow for opsonization.

Meanwhile, the THP-1 cells were prepared by first counting the number of cells and diluting them down to 500,000 cells/ml. These were then co- incubated with the opsonized parasite pellet in a volume of 150 μl in duplicate wells for 45 minutes at 37°C in a CO_2 humified incubator. This incubation step allowed for phagocytosis of the opsonised parasites. The non-phagocytosed RBCs were then lysed using a FACs lysis solution (BD Biosciences) at a 1:10 dilution in distilled water for 10 minutes. Lysis was stopped by the addition of 50 μl of cold PBS (-Ca2+, -Mg2+) 2% FBS and 0.02% $NaNO_3$ (FACS Buffer). The plates were spun down at 350 g for 3 minutes at 4°C. After three washes with 150 μl of cold FACS

buffer the cells were fixed in 150 µl cold 2 % paraformaldehyde in PBS and left on ice before acquiring. Samples were also kept at 4°C if acquisition was to be done the following day.

Acquisition was done using FACSCalibur flow cytometer where THP-1 cells were first gated based on forward scatter (FSC) and side scatter (SSC). A gate was then set on FL2 channel to allow for gating of phagocytosed versus non-phagocytosed THP-1-cells. 10,000 of ethidium bromide-positive THP-1 cells were then acquired at the rate of 200-300 cells /second. The controls in this assay were a polyclonal rabbit anti-human RBC IgG antibody ((ABCAM ab34858), non-immune rabbit IgG control and one well with no parasite (THP-1 alone control). The average proportion of positive cells was calculated by Flowjo for each duplicate wells at different concentrations for the different test antibodies. The negative control (Rabbit IgG) was then subtracted from each of these reads. Final data was expressed as a percentage of the positive control (anti-human RBC IgG antibody). A working protocol is shown in appendix 8.3.

2.3.13 Statistical methods

Comprehensive details of the specific statistical methods are described in each of the results chapters.

CHAPTER 3

Preliminary characterization of *Pf*EMP1 variants associated with rosetting in field isolates

3.1 INTRODUCTION

In *Pf*EMP1 studies, the most abundant *var* gene transcript(s) found at the ring stage of the asexual parasite is thought to be expressed and therefore reflective of adherent specificity of the parasite. However, there is extensive intergenomic and intragenomic variation, as well as high rates of recombination and mosaicism surrounding the study of *var* genes in both laboratory and field isolates (Ward, Clottey et al. 1999; Taylor, Kyes et al. 2000; Kraemer, Kyes et al. 2007; Bull, Buckee et al. 2008; Zilversmit, Chase et al. 2013). The promoter or upstream (Ups) sequence which is fairly conserved has nonetheless been frequently used to classify *var* genes in clinical isolates (Voss, Thompson et al. 2000; Lavstsen, Salanti et al. 2003; Kraemer and Smith 2006). The other approach in the study of *var* genes, which will be used in this chapter, is based on the first duffy binding-like domain (DBLα) of the *Pf*EMP1 and a set of degenerate primers commonly known as universal primers (Taylor, Kyes et al. 2000) have successfully been used to amplify a small region of the domain called the "tag" in both field and laboratory isolates (Kyes, Taylor et al. 1997; Ward, Clottey et al. 1999; Taylor, Kyes et al. 2000). Classification of tag sequences has made DBLα a target of *var* gene transcription studies and has allowed functionally relevant data on *var* genes to be obtained in clinical isolates (Bull, Berriman et al. 2005; Bull, Kyes et al. 2007; Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012).

With rosetting being one of the important adhesion phenotypes associated with severe malaria, studies on *var* gene transcription focusing specifically on rosetting isolates have been done (Rowe, Moulds et al. 1997; Vigan-Womas, Lokossou et al. 2010; Albrecht, Moll et al. 2011; Ghumra, Khunrae et al. 2011). Such studies have led to the identification and characterization of *Pf*EMP1 variants associated with rosetting in several laboratory isolates (Rowe, Moulds et al. 1997; Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011; Ghumra, Semblat et al. 2012). For field isolates, expression profiling of the DBLα tags suggests that distinct sub-groups of *var* genes are implicated in rosetting in natural parasite populations (Kyriacou, Stone et al. 2006; Normark, Nilsson et al. 2007; Bull, Buckee et al. 2008; Warimwe, Fegan et al. 2012) (Bull, Berriman et al. 2005) (Kaestli, Cockburn et al. 2006). However, it is important to note that field isolates unlike laboratory isolates are under intense pressure by host immune responses. The effects of *in vitro* adaptation of patient isolates to laboratory populations may therefore have different selective forces from those affecting the parasite in its native state hence changing the transcription profile of *var* genes (Peters, Fowler et al. 2007). There is therefore need for more information on sequence diversity and transcription profiles of *P.falciparum* parasites especially on culture-adapted field isolates. Caution must however be taken when performing transcription analysis by DBLα RT-PCR on mid/late rings due to the presence of conserved *var* genes such as *var*1csa which have been shown to be constitutively expressed in parasite populations regardless of the phenotype (Winter, Chen et al. 2003; Kyes, Christodoulou et al. 2007). Although transcription of *var1*csa occurs at pigmented trophozoite stage, it is important that such sequences be identified and removed during analysis of *var* gene transcripts, especially if the RNA was extracted from a not so well synchronized culture.

This chapter describes the sequence diversity and transcription profiles of *var* gene transcripts from five culture-adapted field isolates from Kilifi, Kenya. The parasites were obtained from children who presented to Kilifi District Hospital with different syndromes of malaria and varied levels of rosetting frequencies (Chapter 2, Table 2.1). Transcription patterns were compared at the time of disease (acute stage) and after several rounds of rosette selection. Similarly, a comparison was done between isogenic rosette positive (R+) and rosette negative (R-) populations.

3.2: CHAPTER AIMS

The specific aims of this chapter were:-

1. To adapt five clinical isolates into *in vitro* culture conditions and to enrich for rosettes.
2. To clone and sequence *var* genes transcribed by these isolates.
3. To compare sequence diversity and *var* gene transcription profiles before and after *in vitro* adaptation and between isogenic rosette positive (R+) and rosette negative (R-) populations.

3.3: MATERIALS AND METHODS

The methods used in this chapter have been described in details in Chapter 2. They include: - adaptation of field isolates into *in vitro* culture (section 2.3.2), rosette selection (section 2.3.4), extraction of RNA, generation of cDNA, amplification of DBLα tag, sequencing and classification of DBLα tag sequences (all in section 2.3.9). *Mycoplasma* PCR and parasite genotyping were also carried out as described in sections 2.3.7 and 2.3.8 respectively. Sequence analysis on the DBLα tag sequences were done by the Cys/PoLV classification method as

described by Bull *et al.,* 2005 (Bull, Berriman et al. 2005). Details of the classification method have been discussed in Chapter 1 section 1.3.3.2.

Using samples that were being received in an Immunology study at Kilifi (Chapter 2, section 2.2), (Appendix 8.1, Protocol SSC 1131), five *P.falciparum* positive samples were selected from children who presented with different syndromes of malaria and had intermediate levels of rosetting (Chapter 2, Table 2.1). The starting rosetting frequency was determined during the first trophozoite stage *in vitro*. Adaptation into *in vitro* culture was then done as previously described (section 2.3.2). The parasites were rosette-selected weekly by gelatin flotation method (section 2.3.4) to obtain isogenic rosetting (R+) and non-rosetting (R-) sub-populations. Figure 3.1 below illustrates the process of rosette selection by gelatin flotation method done on the five isolates. This was routinely done until the highest possible % RF was obtained for each isolate or above 60%. Ring stage parasites (about 16-20 hours post invasion) were then obtained and the pellet solubilised in Trizol for RNA extraction. This was followed by cDNA preparation, amplification of DBLα tag, sequencing and transcript profiling (Chapter 2, section 2.3.9). Genomic DNA (gDNA) from each sample was also sequenced and *var* gene profiles were used in checking for primer bias in the transcripts. During the process of *in vitro* adaptation and rosette selection, the parasites were genotyped for MSP1/MSP2 alleles (Chapter 2, section 2.3.8) to compare the parasite genotypes before and during the process of adaptation and selection. Throughout this work all cultures were routinely tested for *Mycoplasma* contamination as described in Chapter 2, section 2.3.7.

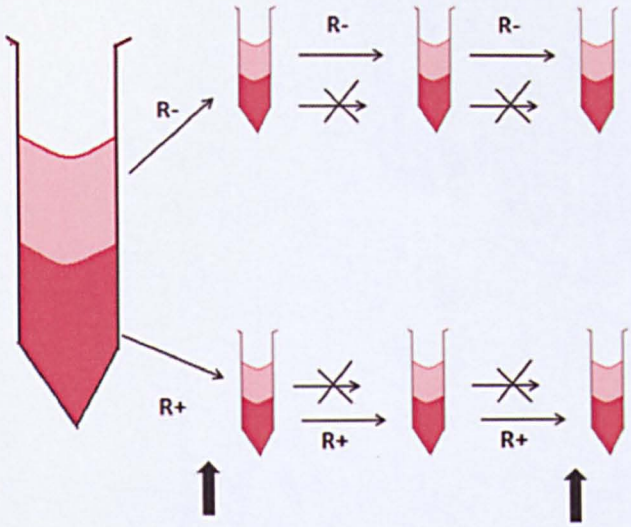


Figure 3.1: *An illustration of the process of rosette selection on culture-adapted field isolates using the gelatin flotation method (Handunnetti, Gilladoga et al. 1992). After the initial gelatin selection, the upper layer which contained rosette negative parasites (R-) was obtained and re-cultured separately for subsequent enrichment of the R- population as shown. The isogenic rosette positive parasites (R+) in the bottom layer were equally re-cultured separately and enriched for rosettes. The process was repeated until the highest and lowest possible % RF was obtained for the R+ and R- respectively. The black arrows at the bottom represent the time-points at which Trizol sample was collected for RNA extraction. To ensure that the R+ population was also knob positive (K+) rosette selection by gelatin method alternated with selection for knob positive parasite (K+) using heparin/gelatin as described in Chapter 2, section 2.3.4.*

3.4: RESULTS

Five field isolates namely 9197, 9106, 8211, 9166 and 8148 were successfully adapted into *in vitro* culture. Details of clinical information of the isolates at the time of disease are listed in Chapter 2 Table 2.1. The isolates took different

lengths of time to exhibit a steady growth and multiplication rate. However, by the 20th cycle, all samples were considered adapted and were each frozen down in more than 5 aliquots that could be thawed out and re-cultured at ease. Due to persistent *Mycoplasma* contamination, sample 9166 was later excluded from this analysis. The results presented here are for the remaining 4 field isolates: - 9197, 9106, 8211 and 8148.

3.4.1: PCR genotyping using merozoite surface protein 1 and 2 (MSP1 and MSP2) markers in acute and rosette selected parasites

It is well known that majority of *P.falciparum* field isolate infections contain multiple clones (Tanner, Beck et al. 1999) some of which could be lost in the process of *in vitro* adaptation. MSP1/MSP2 genotyping was therefore done prior to *var* gene transcriptional profiling to compare the parasite genotypes at acute stage (Figure 3.2, left side gels) and after rosette selection (Figure 3.2, right side gels). The genotypes for R+ and R- populations were also compared. Genes for three allelic variants were amplified: - MSP2 (IC allele), MSP2 (FC allele) and MSP1 (Mad20 allele) (Snounou, Zhu et al. 1999). PCR details are described in Chapter 2 section 2.3.8.

In sample 9197, the R+ and R- rosette-selected samples had fragments of slightly different size for the MSP2-IC allele but this was missing in the acute sample suggesting that they are all different clones. For the FC allele of the same isolate, a faint band approximately 330bp was detected in acute sample but this was lost on adaptation. The Mad20 allele of 9197 however showed identical fragments for both the acute and the two rosette-selected parasites.

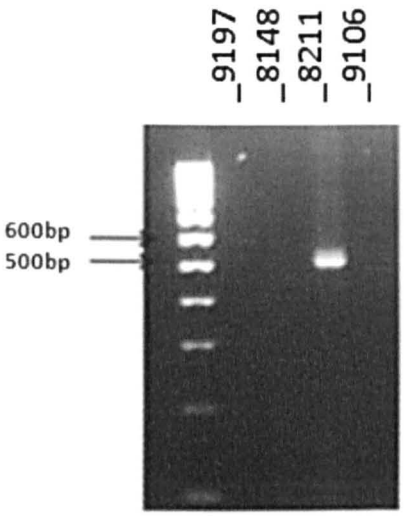
In sample 8148, no fragment was detected in both the acute and rosette selected samples for the IC allele. For the FC allele, the acute sample had a similar band pattern to the R+ and not the R- population. The latter had lost the approximately 420 bp fragment. At the Mad20 allele, the R+ and R- parasites had similar band patterns but which were different from the acute sample.

In sample 8211, the bands for IC allele in the acute and rosette-selected samples were of the same size. However, the R+ and R- bands were of different intensities. This could have been due to less DNA template in the latter at the time of genotyping. The FC allele on the other hand had no bands in both the acute and the selected parasites. However, results on Mad20 allele of the same sample suggests that the R+ and R- were of different genotypes due to the slight difference in band size.

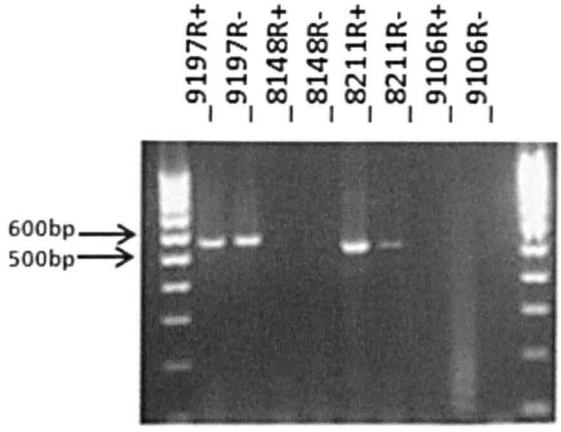
Finally, sample 9106 showed no product for the IC allele for both the acute and selected parasites. However, on the FC and Mad20 alleles, the R+ and R- populations showed slight differences in the sizes of the bands suggesting differences in genotypes.

Overall, results from parasite genotyping suggest that the process of *in vitro* adaptation of field isolates and the subsequent rosette selection into R+ and R- was accompanied by changes in clones.

MSP2 (IC allele)

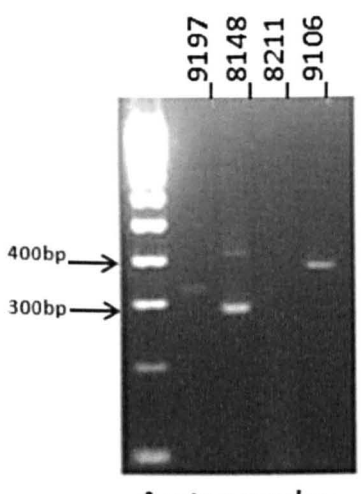


Acute samples

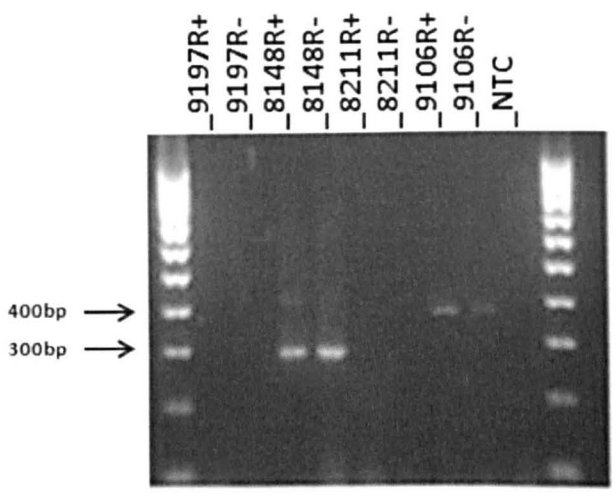


Rosette-selected samples

MSP2 (FC allele)



Acute samples



Rosette-selected samples

MSP1 (Mad20 allele)

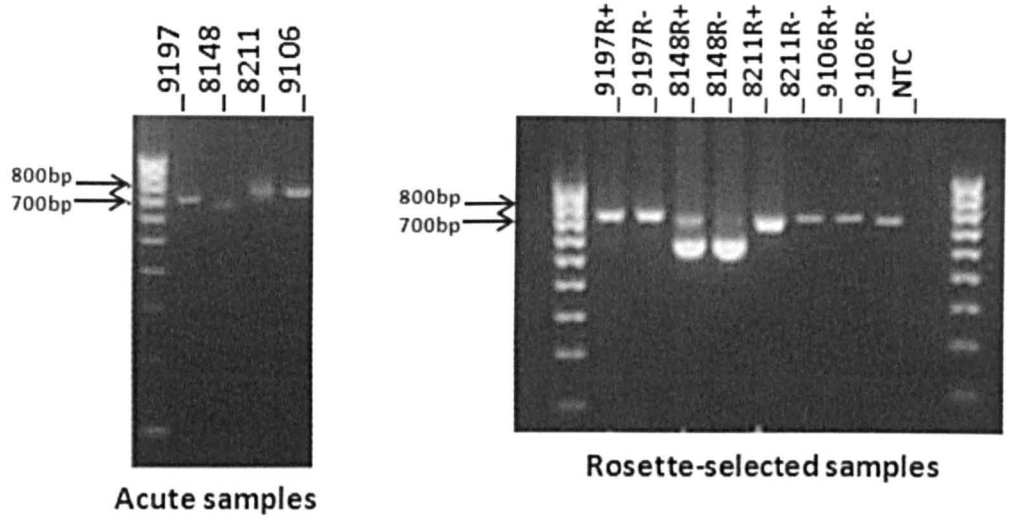


Figure 3.2: *Ethidium bromide-stained agarose gels showing PCR products from genotyping of polymorphic alleles in merozoite surface proteins. These were:-IC allele (MSP2), FC allele (MSP2) and Mad20 (MSP1). Genotypes for acute samples (gels on the left panel) were compared to rosette selected R+ and R- samples (gels on the right panel). The order of the sample on the gels was 9197, 8148, 8211 and 9106. The molecular weight marker used was a 100bp ladder (New England Biolabs). NTC represents a no-template control that was included in all PCRs.*

3.4.2: Rosette selection by gelatin flotation

Weekly enrichment of rosettes by gelatin flotation (Chapter 2, section 2.3.4) allowed for maintenance of increasing levels of rosettes in some but not all isolates. It is important to note that the increase was not steady and in most cases took several rounds of selection to see a significant increase in the % RF. Only 3 out of the 4 samples (9197, 8211 and 8148) had their rosette frequencies enriched

to high levels with an average % RF of about 60% (Figure 3.3). Sample 9106 did not achieve high % RFs even after several rounds of selection. However, the initial rosetting frequency for this sample was low (9%) compared to the rest of the samples. It could also be that these parasites have inherently low rosetting frequencies that could not be enriched even after several rounds of selection.

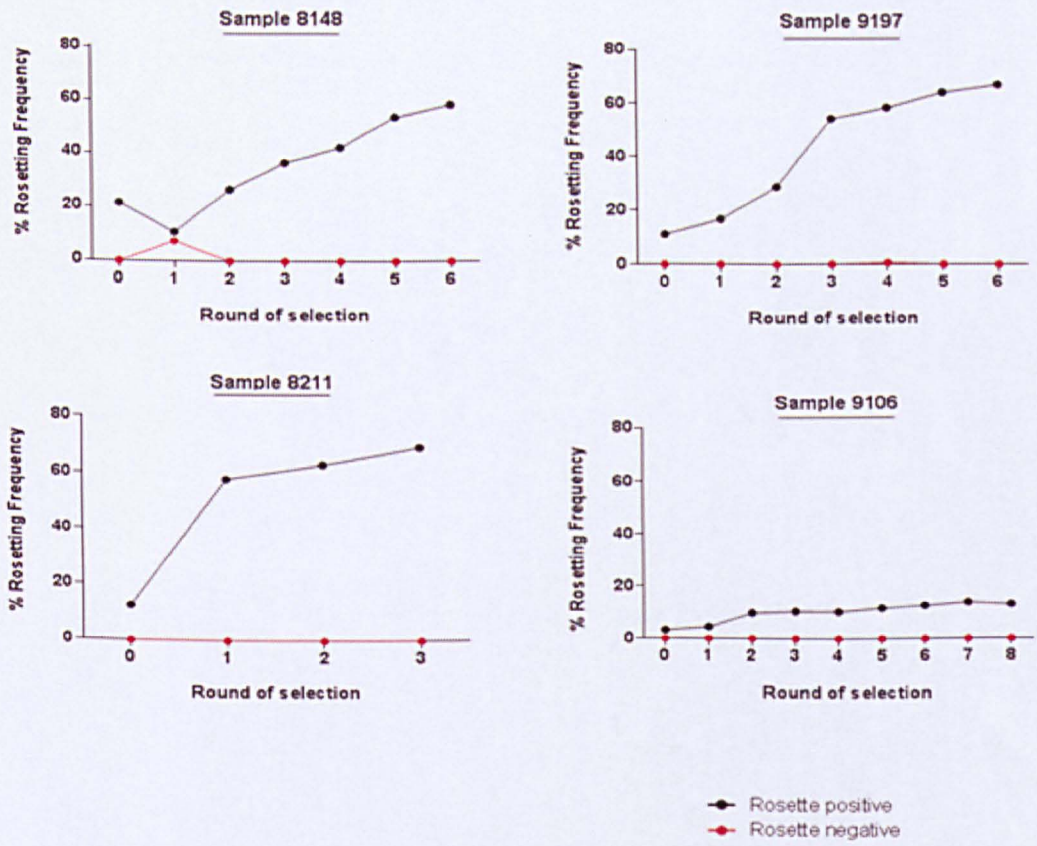
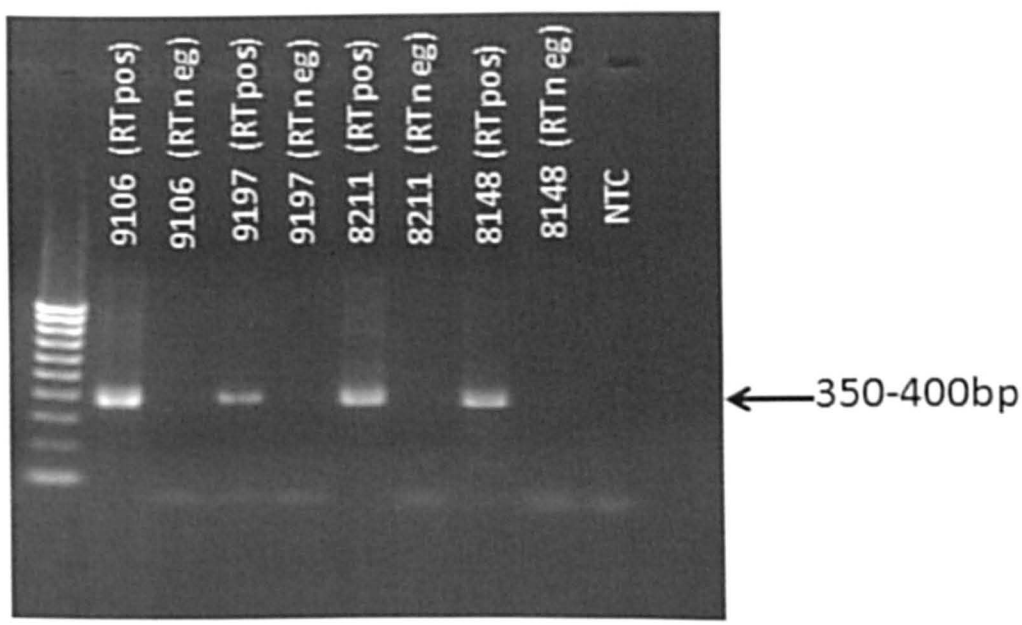


Figure 3.3: *Rosette selection of 4 isolates into R+ (black line) and R- (red line) populations by gelatin flotation method (section 2.3.4 of chapter 2). The graphs show changes in % RF (y axis) for the two isogenic populations during rounds of selection (x axis). Selection was done until the highest possible and the lowest % RF was attained for each R+ and R- respectively. RNA samples for the transcriptional profiling were collected at the final point of selection for each of the samples.*

3.4.3: Amplification of DBLα tag region of the PfEMP1

Amplification of the DBLα tag region of the *Pf*EMP1 and transcriptional profiling (details in Chapter 2, section 2.3.9) was done on RNA collected from the ring stage of R+ and R- populations at the end of rosette selection. For the acute samples, the RNA was prepared from the ring stage sample that was frozen straight from the patient's arm. cDNA was then made from all the RNA samples and amplification done using modified DBLαAF and DBLαBR primers which target a 300-400bp region between the homology blocks D and H of the DBLα domain (Bull, Berriman et al. 2005). Results of the PCR amplification for the reverse transcribed sample (RT pos) are shown in Figure 3.4. For each of the amplifications, a control was included that was not treated with reverse transcriptase enzyme denoted (RT neg) to ensure that there was no contaminating DNA in the RNA preparation.

Gel 1: Acute samples



Gel 2: Rosette selected samples

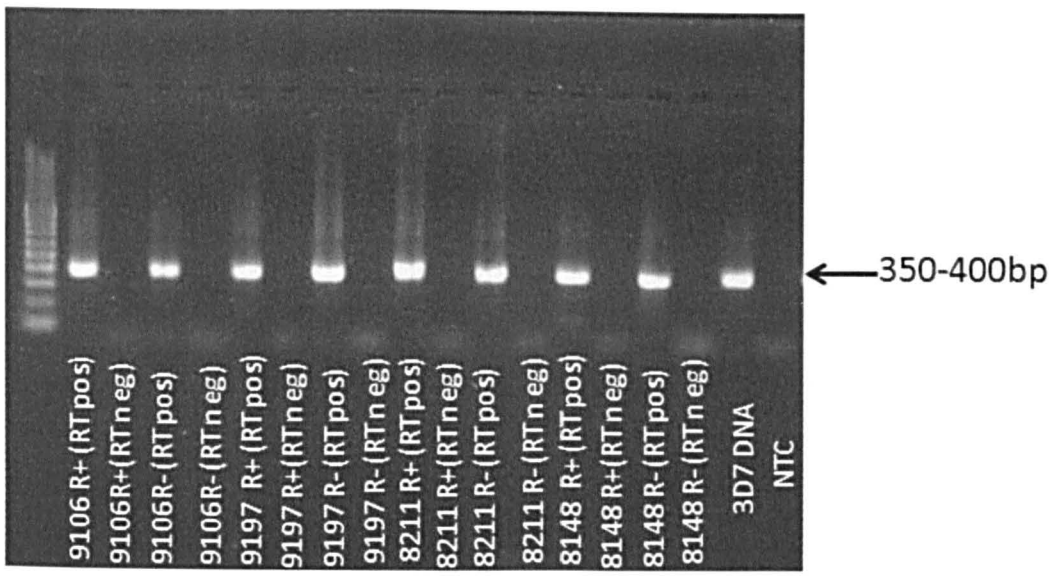


Figure 3.4: *2% agarose gels stained with ethidium bromide showing PCR products from amplification of DBLα tag of the cDNA samples. The product sizes range from between 350-400bp. Gel 1 shows amplification products from acute samples while Gel 2 shows the PCR products for isogenic R+ and R-. Samples were treated with and without reverse transcriptase enzyme represented as "RT pos" and "RT neg" respectively. DNA from 3D7 was amplified as a positive control and a no-template control (NTC) was included as a negative control. The molecular weight marker used was a 100bp ladder (New England Biolabs).*

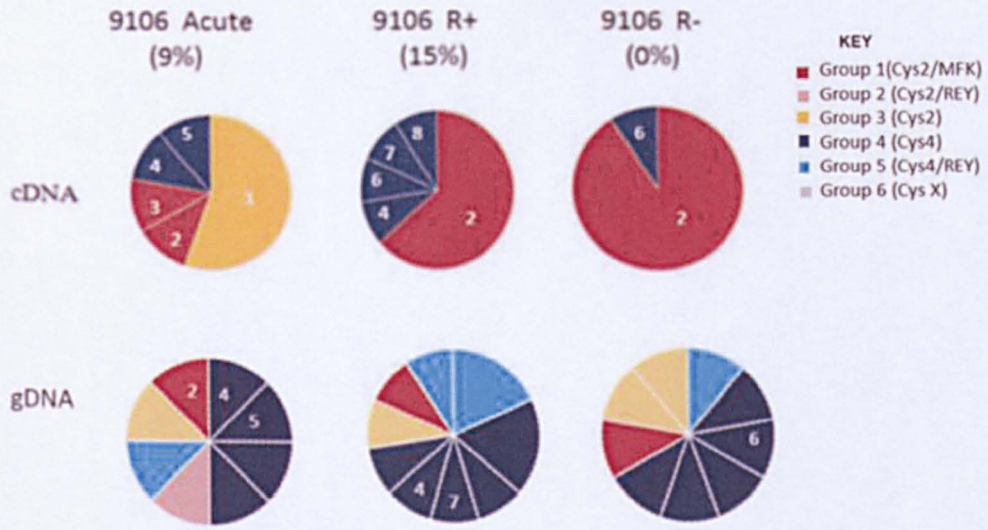
3.4.4 Sequence analysis and var gene transcription profiling

Sequencing of the cloned DBLα tag was done using the capillary method (Big Dye Terminator v3.1 cycle sequencing kit-Applied Biosystems). The sequences were then analyzed by cys/PoLV (CP) classification method (Chapter 1, section 1.3.3.2) in which the six CP groups were represented by different colours (Figure 3.5, legend). Identical sequences herein referred to as sequence "types" were

identified for each isolate and assigned arbitrary identity numbers (Figures 3.5-3.8) which were then assigned to one of the CP groups. However, it was possible that two different sequence types could fall within the same CP group, and therefore the two different slices of the pie would be represented by the same colour. The number of slices in each pie chart therefore shows the number of unique sequence types for each sample and the size represents their relative proportion to the total number of sequences. Since the profiling involved few samples, identification of sequence types was done by a manual count of the unique sequences following clustal W alignment on Bio-edit Sequence alignment editor. Sequences from gDNA sequences were also profiled in a similar manner. The aim of including the gDNA profiles was to check for primer bias. For instance, if the dominant cDNA sequence was also the dominant gDNA sequence, this would be indicative of primer bias. In Figure 3.5-3.8, only the gDNA sequences types that were identical to any of the transcribed sequences are labelled. All sequences in FASTA format are shown in the appendix 8.4.

For each isolate, a *var* gene thought to be involved in rosetting was identified as the dominant sequence type transcribed by the R+ population, but which was also rare or absent in the R- population. In 9106 (Figure 3.5), the dominant gene in R+ (sequence_2, belonging to CP1 group -63.64%) was also dominant in R- (90.91%) and therefore was ruled out as the gene responsible for rosetting. The gene was also present in the acute sample. It was however interesting that sequence_2 was expressed in such a high proportion despite the low rosetting frequency in 9106. Sequence_2 bears the MFK motif (CP1), a group A type that has previously been linked to rosetting and severe disease (Bull, Berriman et al. 2005; Bull, Kyes et al. 2007; Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012). Whether selection away from rosetting led to the enrichment of another virulent *var* gene

remains unknown. The possibility of primer bias was ruled out since sequence_2 was not dominantly expressed in gDNA of R+ and R-.

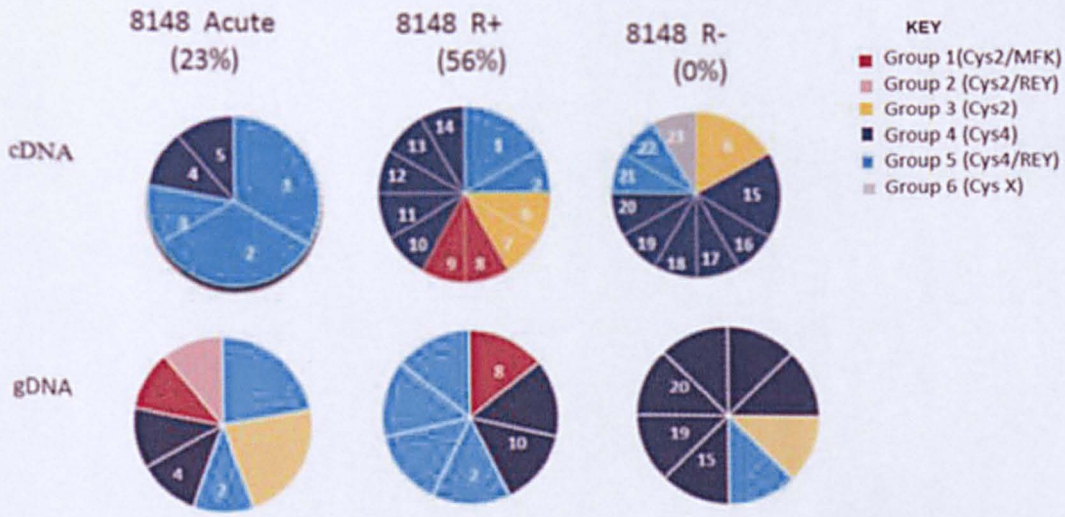


		Number of sequences (proportion)
9106_Acute	**Sequence Types**	
	9106_sequence_ 1	5 (55.56 %)
	9106_sequence_ 2	1 (11.11 %)
	9106_sequence_ 3	1 (11.11 %)
	9106_sequence_ 4	1 (11.11 %)
	9106_sequence_ 5	1 (11.11 %)
	Total	9 (100 %)
9106R+	9106_sequence_ 2	7(63.64 %)
	9106_sequence_ 4	1 (9.09 %)
	9106_sequence_ 6	1 (9.09 %)
	9106_sequence_ 7	1 (9.09 %)
	9106_sequence_ 8	1 (9.09 %)
	Total	11 (100 %)
9106R-	9106_sequence_ 2	10 (90.91 %)
	9106_sequence_ 6	1 (9.09 %)
	Total	11 (100 %)

Figure 3.5: *Var gene transcription profiles of the acute samples and the isogenic R+ and R- populations in sample 9106. The pie charts show the cDNA and gDNA*

var gene profiles for the acute, R+ and R- samples. The different sequences and their proportion of the total sequences are listed on the table below the charts. The different colours represent the CP groups to which the unique sequence types belong. The labelled sequences at the gDNA level represent only those sequences that were identical to a cDNA sequence, as a way of checking for any form of primer bias.

Sample 8148 (Figure 3.6), had two dominant sequences at acute stage (sequence_1 and 2) and which were in equal proportions (33.33%). The two sequences also came up in the R+ selected sample. Although sequence_1 was dominant in R+ and was not seen in R-, it could not convincingly be referred to as the gene responsible for rosetting due to the small number of sequences.



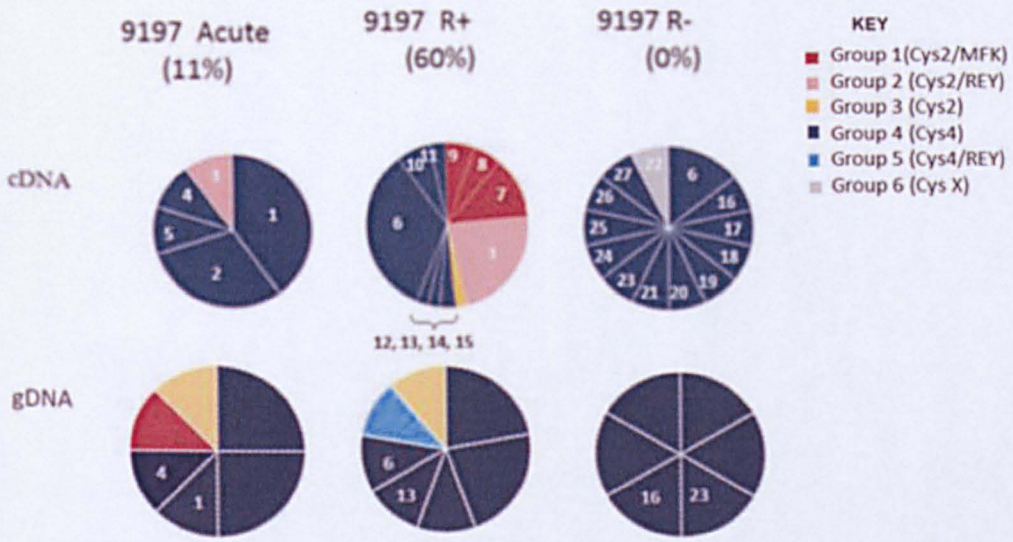
8148_Acute	Sequence Types	Number of sequences (proportion)
	8148_sequence_ 1	3 (33.33%)
	8148_sequence_ 2	3 (33.33%)
	8148_sequence_ 3	1 (11.11%)
	8148_sequence_ 4	1 (11.11%)
	8148_sequence_ 5	1 (11.11%)
	Total	9 (100%)

8148R+	8148_sequence_1	2 (16.67%)
	8148_sequence_2	1 (8.33%)
	8148_sequence_6	1 (8.33%)
	8148_sequence_7	1 (8.33%)
	8148_sequence_8	1 (8.33%)
	8148_sequence_9	1 (8.33%)
	8148_sequence_10	1 (8.33%)
	8148_sequence_11	1 (8.33%)
	8148_sequence_12	1 (8.33%)
	8148_sequence_13	1 (8.33%)
	8148_sequence_14	1 (8.33%)
	Total	12 (100 %)
8148R-	8148_sequence_6	2 (18.18 %)
	8148_sequence_15	2 (18.18 %)
	8148_sequence_16	1 (9.09 %)
	8148_sequence_17	1 (9.09 %)
	8148_sequence_18	1 (9.09 %)
	8148_sequence_19	1 (9.09 %)
	8148_sequence_20	1 (9.09 %)
	8148_sequence_21	1 (9.09 %)
	8148_sequence_22	1 (9.09 %)
	8148_sequence_23	1 (9.09 %)
	Total	**11**

Figure 3.6: *Var gene transcription profiles of the acute samples and the isogenic R+ and R- populations in sample 8148. The pie charts show the cDNA and gDNA var gene profiles for the acute, R+ and R- samples. The different sequences and their proportion of the total sequences are listed on the table below the charts. The different colours represent the CP groups to which the unique sequence types belong. The labelled sequences at the gDNA level represent only those sequences that were identical to a cDNA sequence, as a way of checking for any form of primer bias.*

Sample 9197 (Figure 3.7) also had 2 dominant CP4 sequences (sequence_1 and 2) at acute stage. In the R+ sample, sequence_6 was dominant (33.33%) but was also dominant in R- (15.38 %). The second dominant sequence in R+

(sequence_3) was not present in R- and could be possibly be the gene responsible for rosetting. Sequence_3, which belongs to CP2, was also present in the acute sample although not as a dominant gene. There were more group A sequence types in R+ and none in the R- in support of previous association between Group A genes and rosetting.

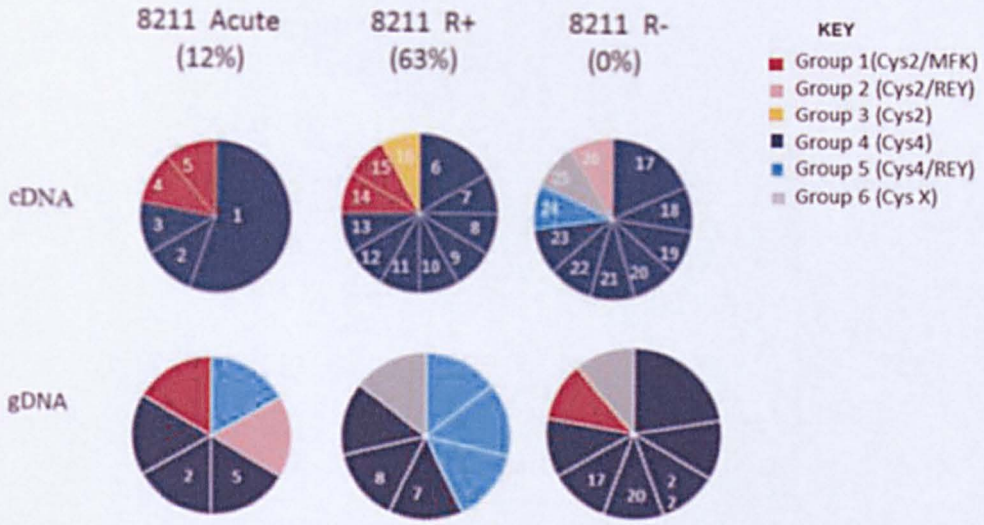


	Sequence Types	Number of sequences (proportion)
9197_Acute	9197_sequence_ 1	4 (40 %)
	9197_sequence_ 2	3 (30%)
	9197_sequence_ 3	1 (10%)
	9197_sequence_ 4	1 (10%)
	9197_sequence_ 5	1 (10%)
	Total	10 (100%)
9197R+	9197_sequence_ 6	9 (33.33 %)
	9197_sequence_3	6 (22.22%)
	9197_sequence_7	3 (11.11%)
	9197_sequence_ 1	2 (7.41 %)
	9197_sequence_8	2 (7.41 %)
	9197_sequence_9	2 (7.41 %)
	9197_sequence_10	1 (3.70%)
	9197_sequence_11	1 (3.70%)
	9197_sequence_12	1 (3.70%)
	9197_sequence_13	1 (3.70%)
	9197_sequence_14	1 (3.70%)
	Total	29(100%)

9197R-	9197_sequence_ 6	2 (15.38%)
	9197_sequence_ 15	1 (7.69%)
	9197_sequence_ 16	1 (7.69%)
	9197_sequence_ 17	1 (7.69%)
	9197_sequence_ 18	1 (7.69%)
	9197_sequence_ 19	1 (7.69%)
	9197_sequence_ 20	1 (7.69%)
	9197_sequence_ 21	1 (7.69%)
	9197_sequence_ 24	1 (7.69%)
	9197_sequence_ 25	1 (7.69%)
	9197_sequence_ 26	1 (7.69%)
	9197_sequence_ 27	1 (7.69%)
	Total	**13**

Figure 3.7: *Var gene transcription profiles of the acute samples and the isogenic R+ and R- populations in sample 9197. The pie charts show the cDNA and gDNA var gene profiles for the acute, R+ and R- samples. The different sequences and their proportion of the total sequences are listed on the table below the charts. The different colours represent the CP groups to which the unique sequence types belong. The labelled sequences at the gDNA level represent only those sequences that were identical to a cDNA sequence, as a way of checking for any form of primer bias.*

Sample 8211 (Figure 3.8), was the most heterogenous of the four with varied sequence types in acute, R+ and R- samples that were all expressed in low proportions. There was no clear dominant gene in R+ that could be linked to rosetting.



	Sequence Types	Number of sequences (proportion)
8211_Acute	8211_sequence_ 1	5 (55.56%)
	8211_sequence_ 2	1 (11.11%)
	8211_sequence_ 3	1 (11.11%)
	8211_sequence_ 4	1 (11.11%)
	8211_sequence_ 5	1 (11.11%)
	Total	9 (100%)
8211R+	8211_sequence_ 6	2 (16.67%)
	8211_sequence_ 7	1(8.33%)
	8211_sequence_ 8	1(8.33%)
	8211_sequence_ 9	1(8.33%)
	8211_sequence_ 10	1(8.33%)
	8211_sequence_ 11	1(8.33%)
	8211_sequence_ 12	1(8.33%)
	8211_sequence_ 13	1(8.33%)
	8211_sequence_ 14	1(8.33%)
	8211_sequence_ 15	1(8.33%)
	8211_sequence_ 16	1(8.33%)
	Total	12 (100%)
8211R-	8211_sequence_ 17	2 (18.18 %)
	8211_sequence_ 18	1 (9.09 %)
	8211_sequence_ 19	1 (9.09 %)
	8211_sequence_ 20	1 (9.09 %)
	8211_sequence_ 21	1 (9.09 %)
	8211_sequence_ 22	1 (9.09 %)
	8211_sequence_ 23	1 (9.09 %)

8211_sequence_ 24	1 (9.09 %)
8211_sequence_ 25	1 (9.09 %)
8211_sequence_ 26	1 (9.09 %)
Total	10 (100 %)

Figure 3.8: *Var gene transcription profiles of the acute samples and the isogenic R+ and R- populations in sample 8211. The pie charts show the cDNA and gDNA var gene profiles for the acute, R+ and R- samples. The different sequences and their proportion of the total sequences are listed on the table below the charts. The different colours represent the CP groups to which the unique sequence types belong. The labelled sequences at the gDNA level represent only those sequences that were identical to a cDNA sequence, as a way of checking for any form of primer bias.*

There were possible limitations in this sequence analysis approach, some of which were addressed. These included: -

i. Presence of *var*1 sequences that would constitutively be expressed regardless of the phenotype and hence confound the analysis (Winter, Chen et al. 2003).

ii. Possible primer bias (Taylor, Kyes et al. 2000; Kraemer, Gupta et al. 2003)

iii. Rapid switching off of the Group A s *in vitro* as described in Peters *et al.,* 2007 (Peters, Fowler et al. 2007) and Frank *et al.*,2007 (Frank, Dzikowski et al. 2007).

iv. Inability to select and identify rosetting genes of clinical importance or those expressed at the time of disease.

v. Limited number of sequences.

i) Checking for presence of var1 sequences

Var1 sequences have been shown to be constitutively expressed in pigmented trophozoites regardless of the parasite phenotype (Winter, Chen et al. 2003; Kyes, Christodoulou et al. 2007). Prior to compiling the final transcript profile, all sequences were checked for the presence of two types of *var* 1 sequence which could otherwise have confounded the analysis. The first is in CP1 (MFK^+ REY^-) and was identified using the motif NVHDKVEK/TGLREVF and corresponded to *var* 1 genes from four other isolates: -TM180, TM284 (Rowe, Kyes et al. 2002), FCR3, and HB3 (Kraemer, Kyes et al. 2007). The second falls in cys/PoLV group 2 (MFK^- REY^+) and was identified using the motif APNKEKIKLEENLKK corresponding to *var* 1 from 3D7 and "common" (Winter, Chen et al. 2003). A perl script written by Pete Bull that could identify the *var* 1 motif was run on all samples. None of the sequences generated from the acute and rosette-selected samples contained the *var* 1 sequence.

ii) Assessing primer bias using gDNA

Primer bias was checked by comparing the dominant sequence of the gDNA profiles with that of the transcript profiles. Identical sequences between the dominant transcript sequence and a dominant gDNA sequence would be indicative of bias towards amplification of that gene. The pie charts for the gDNA sequences (Figure 3.5-3.8) show only those sequences that were identical to any of the cDNA sequences. There was no evidence of primer bias in any of the four isolates.

iii) Were any of the var genes in the acute samples present in the rosette selected samples?

To check whether sequences in the acute sample were maintained after *in vitro* adaptation and rosette selection, acute sequences were compared against those from rosette selected lines. It is assumed that any dominant sequence at acute stage was responsible for the clinical syndrome exhibited by the patient at the time of disease. In 3 of the isolates (9106, 8148 and 9197), some transcript sequence present in the acute sample was also present in the rosette selected sample. Although they were not necessarily the dominant sequences at acute stage, they represent sequences that may have been important for the disease process. While the dominant CP groups were maintained in 9197 and 8211, the actual individual sequence types were different. Two samples i.e. 8148 and 9106 showed a major switch of genes from the acute to the rosette selected lines, with only a few acute sequences coming up in rosette selected samples. Although the 9106R+ could not be selected to high rosetting frequencies, an interesting finding was that the dominant sequences in R+ and R- were exactly the same i.e 9106_sequence 2. Furthermore, despite the inability to select 9106R+ to high % RF (Figure 3.3), the sequence_2 contained one of the motifs DKVEKG termed "H3", classified by Normark and colleagues (Normark, Nilsson et al. 2007) as a high rosetting motif. Primer bias was ruled out since none of the gDNA sequences were identical to this sequence.

iv) Inability to select and identify rosetting genes of clinical importance

Due to the differences in selective forces between the parasites in *in vivo* and *in vitro* conditions, it is possible that the process of adaptation and subsequent process of rosette selection of parasites described in this chapter resulted in

expression of *Pf*EMP1 variants that are not clinically relevant. Previous studies have reported that the effects of *in vitro* adaptation of patient isolates to laboratory populations resulted in changes in transcription profiles of *var* genes (Peters, Fowler et al. 2007). Although clinical relevance was not directly tested, an indirect way would be to check if any of the dominant sequences at acute stage (presumably those responsible for the disease) were also dominantly expressed after selection as described in (iii) above.

v) Limited number of sequences

For all the four isolates, the number of good quality sequences that were analyzed ranged between 9-29. The probability of picking a dominant sequence could be limited by few sequences. However, the acute samples in 3 of the 4 isolates (9106, 8211 and 9197) were further sequenced by next generation sequencing technique (454 platform) which gives more sequences. A comparison of the transcription profiles by capillary method versus 454 method (section 3.4.5 below), showed the same dominant sequence was picked by both methods.

3.4.5 Comparison of acute transcript's sequences by capillary method versus next-generation 454 sequencing method

Next generation sequencing techniques such as the 454 platform have been shown to be efficient in sequencing of polymorphic sites, while producing longer reads with deeper coverage (Gandhi, Thera et al. 2012). The transcript sequences of samples 9106, 9197 and 8211 described in this chapter have recently been sequenced in Sanger on the 454-platform. Unlike the capillary method where 5-27 clones were sequenced per isolate, the 454 method had more depth with more

sequences being obtained per isolate (9197=118 sequences, 9106=447 sequences and 8211=521 sequences). Sequences generated by 454 were compared to those obtained by capillary method to determine if the limited number of sequences by the capillary method could have affected the precision of the estimate of proportional abundance. Figure 3.9 shows the transcript profiles for acute samples using both methods. It was encouraging to note that despite the small number of sequences by the capillary method in comparison with the 454 method, the dominant sequences type in all the 3 isolates were identical by the two methods. However, the 454 method was able to pick up more sequences than the capillary method. It is therefore likely that the rosette-selected samples which were all sequenced by the capillary method gave the correct dominant sequence.

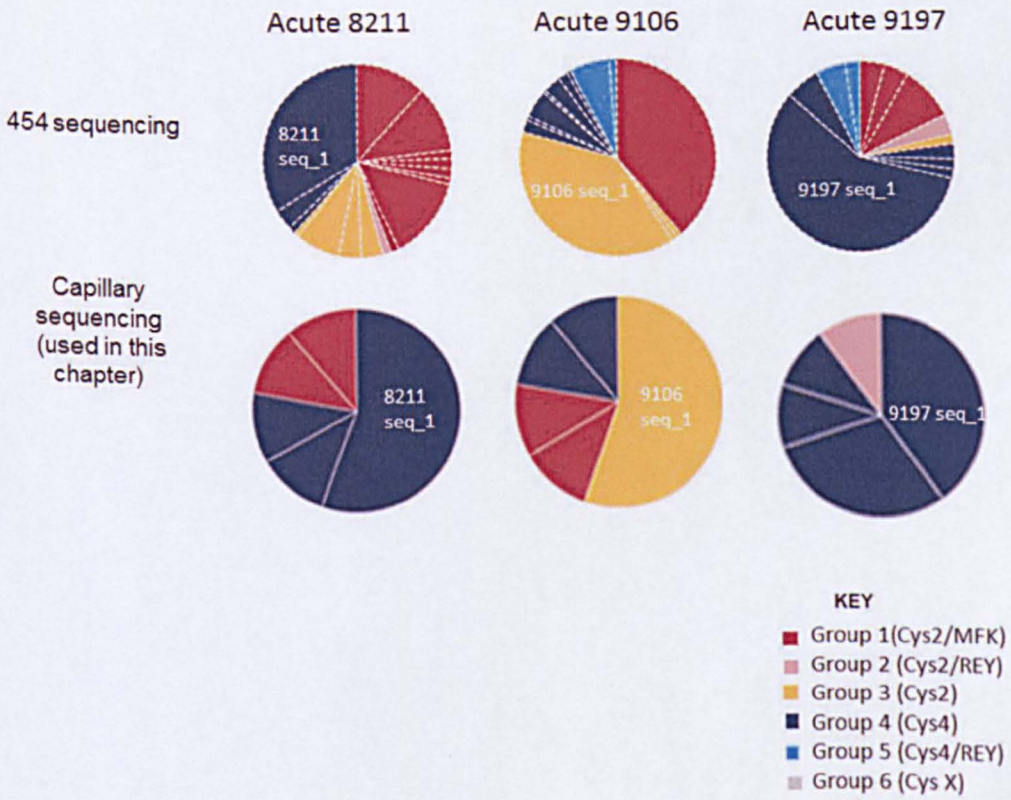


Figure 3.9: *A comparison of transcript profiles by 454 and capillary methods in 3 of the acute cDNA samples. The transcripts were generated from different aliquots of the acute sample. The pie charts show the proportion of unique sequence types to the total number of sequences by each method and colours represent different sequence groups by Cys/PoLV classification method as previously described. The dominant sequences were the same by both methods i.e. 8211_sequence_1, 9106_sequence_1 and 9197_sequence_1. The total numbers of individual sequences for each isolate by 454 were: 8211=521 sequences, 9106=447 sequences and 9197 =118 sequences while the capillary method had between 9-10 sequences per isolate. Despite the difference in number of reads between the two methods, the dominant sequences for each isolate were identical.*

3.5 DISCUSSION

While a lot of rosetting *Pf*EMP1 variants in laboratory strains have been characterised (Rowe, Moulds et al. 1997; Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011), little information is available concerning *var* genes associated with rosetting in field isolates. Many field studies that have looked at *var* gene transcription in rosetting isolates were generally examining the association between genes and severe malaria (Kyriacou, Stone et al. 2006; Normark, Nilsson et al. 2007; Bull, Buckee et al. 2008; Warimwe, Fegan et al. 2012). I aimed to specifically examine the composition of *var* gene transcripts in culture adapted field isolates and their transcription patterns before and after rosette selection. Following successful adaptation of the field isolates into *in vitro* culture, transcriptional profiling of *var* gene sequences was done before and after rosette selection.

Var genes transcribed during acute stage were identified in four field isolates. At this stage however, it was not possible to link any of the *var* genes to rosetting phenotype since all the samples had relatively low % RF (range 9-23%). It was however expected that any of the dominant gene upon rosette selection, and which was present in the acute transcripts, would be potentially linked to rosetting. None of the dominant transcripts in the rosette-selected samples were present in the acute samples. This could have been due to the limited number of clones that were sampled for sequencing. The dominant *var* gene transcribed at the acute stage could have been responsible for severe disease through other mechanisms than rosetting. It is also likely that there is a change of genotypes during the process of *in vitro* adaptation as seen in the MSP1/MSP2 results.

To identify *var* genes responsible for rosetting, transcripts from R+ were compared to those from R- (Figure 3.5-3.8). Putative rosette-mediating variants in each isolate were identified as a dominant sequence type transcribed by the R+ population, and which was rare or absent in the R- population. It was expected that *var* genes responsible for rosetting in 8211, 9197 and 8148 would be easily identified since they were all selected to relatively high % RF ranging from 56-63%. However this was not the case. Only in sample 9197R+ was there a dominant sequence in the CP2 group that was absent in the isogenic R- population. In samples 8211 and 8148, no clear dominant sequence came up in the R+ population. It is possible that the rosette-selected parasite population was heterogenous and contained rosettes that were mediated by different variants.In such a case, a dominant *var* gene may not have been easily identified. This is possible considering that previously characterised IT*var*9 and IT*var*60 *Pf*EMP1 variants, both of which mediate rosetting are present within the IT genome (Rowe, Moulds et al. 1997; Albrecht, Moll et al. 2011). Interestingly, despite not achieving

high % RF, it was the rosette-selected sample 9106 that showed a homogenous transcriptional profiling pattern with a single dominant *var* gene (up to 63.64% CP1) upon selection. The same sequence was also seen in the R- population (90.91%) It remains unknown the importance of high level transcription of this gene.

While *in vitro* systems provide insights into the biological processes of parasites, major differences between *in vivo* and *in vitro P. falciparum* growth conditions have been highlighted, as reviewed by LeRoux *et al.*, 2009 (LeRoux, Lakshmanan et al. 2009). One major difference between *in vivo* and *in vitro* parasites that could have been a caveat in the approach described here is the possibility that immune pressure is exerted on the parasite inside the host while no similar pressure is exerted under *in vitro* conditions. This could potentially alter the expression of *Pf*EMP1 which is thought to be an important immune target in the host. It also implies that the process of *in vitro* adaptation and subsequent rosette selection as described in this chapter could have been selecting for clinically irrelevant variants or that the highly relevant variants which were strongly recognized by the immune response cannot survive *in vitro* conditions. Only a few genes, although not necessarily the dominant ones, were identical in both the acute and rosette selected transcripts.

*Pf*EMP1 variants associated with rosetting in laboratory isolates are mostly of group A type, which is characterised by two cysteine residues (Rowe, Moulds et al. 1997; Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011; Ghumra, Khunrae et al. 2011). In this analysis, only sample 9197 R+ had a Group A var gene (CP2) that could be linked to rosetting. Samples 8148 and 8211 had non-Group A-like genes as the dominant genes. The proportions of these sequences were however not convincingly higher than the rest of the sequences and

therefore they could not be definitively linked to rosetting. It is suggested that genes from Group A have a higher switch off rate *in vitro* than genes from Ups B and C (Peters, Fowler et al. 2007). It is possible that the rosetting field isolates described in this chapter lost some of their expressed Group A genes due to switching during the process of *in vitro* adaptation. This hypothesis may however be questionable since sample 9106 was able to maintain high levels of a Group A sequence *in vitro* in both the R+ and R- populations.

Previous studies have shown that *in vitro* adaptation of malaria parasites in culture could result in loss of clones (Nsobya, Kiggundu et al. 2008; Cheeseman, Gomez-Escobar et al. 2009). In this study, a comparison of parasite clones before and after selection by MSP genotyping showed evidence of loss or change of genotypes in all the 4 isolates. Although the dominant *var* gene transcripts would possibly be from the dominant genotype, it is possible that the comparison of *var* gene transcripts was affected by these differences in parasite genotypes especially in cases where the R+ and R- parasites. This could have greatly contributed to the inability to identify a single dominant gene responsible for rosetting.

In conclusion, sequence diversity and transcription profiles from 4 culture-adapted field isolates were evaluated. Unlike previous work using *P. falciparum* laboratory strains (Ghumra, Semblat et al. 2012), it was not possible to identify a predominant *var* gene in any of the rosette-selected parasites. Later examination of the rosette-selected Kenyan parasites in the Rowe lab suggested that the rosette frequencies of the selected lines were much lower than were initially estimated in Kilifi. The most likely explanation for the failure to identify predominant *var* genes linked to rosetting is that the parasites were not sufficiently well selected to high rosette frequency to give a clear phenotype. Related to this is

the possibility that the rosette selection method using gelatin flotation resulted in retention of a heterogenous parasite population consisting of rosetting parasites and knob-negative non-rosetting parasites, that would hinder clear transcriptional profiling. The culture-adapted field isolate described in chapter 4 and 5 was characterized following cloning by limiting dilution to attempt to obtain highly distinct but isogenic R+/R- lines with a more stable phenotype. Another explanation for the lack of a predominant *var* gene linked to rosetting is the possibility that the Kenyan parasites might rosette using non-PfEMP1 ligands such as rifins or stevors. Further work will be needed to examine this possibility. Another limitation of the work presented here is the lack of depth in the sequencing of R+ and R- clones, which could be addressed in future work either by sequencing more colonies or by using next generation sequencing methods like 454, as was done on the acute samples.

CHAPTER 4

Cloning and sequencing of full-length *Pf*EMP1 in a field isolate, protein expression and raising of antibodies from individual recombinant domains

4.1 INTRODUCTION

Although *Pf*EMP1 is encoded by a large and diverse *var* gene family of about 60 in a haploid genome (Kyes, Horrocks et al. 2001), only one (out of the possible 60) is expressed at a time, reviewed by Scherf *et al.,* 2008 (Scherf, Lopez-Rubio et al. 2008). It is this mutually exclusive expression of a single *var* gene in an infected cell that determines the antigenic, cytoadherent, and virulence phenotypes such as rosetting. Many studies, including this one, aim at identifying *Pf*EMP1 variants that are associated with particular adhesion phenotypes in *P. falciparum* isolates.

4.1.2: Rosette-mediating PfEMP1 variants

Various attempts have previously been made to identify and characterize *Pf*EMP1 variants that are associated with rosetting in *P. falciparum.* Three examples of well characterized rosette-mediating *Pf*EMP1 variants are R29*var*1 (also known as IT*var*9) expressed by the IT/R29 clone (Rowe, Moulds et al. 1997), IT*var*60 in the FCR3S1.2 clone (Albrecht, Moll et al. 2011) and *Var* O in the Palo Alto strain (Vigan-Womas, Guillotte et al. 2008). The GeneBank accession numbers for these variants are: -YI3402, EF158099 and EU9082205 respectively. Following their identification, assays have been done using recombinant proteins or antibodies raised to the variant proteins in order to determine their functional and immunological properties.

First to be identified and characterized was the rosette-mediating variant in IT/R29 clone (Rowe, Moulds et al. 1997). A comparison of Duffy-binding-like domain (DBL-1) transcripts from isogenic rosette positive (R29+) and rosette negative (R29-) parasites showed IT*var*9 gene to be exclusively expressed in R29+ and not in R29-. The full-length IT*var*9 (8.2 kb), a group A *var* gene, was then identified by PCR walking, cloning and sequencing using vectorette libraries (Arnold and Hodgson 1991). Complement receptor 1 (CR1) was shown to be the host receptor on uninfected red cells involved in rosetting in IT/R29.

A second rosetting parasite whose *Pf*EMP1 variant has been characterized is FCR3S1.2 (Chen, Barragan et al. 1998), a clone derived from FCR3S1 by micromanipulation (Fernandez, Treutiger et al. 1998). An initial report (Chen, Barragan et al. 1998) had shown that rosetting in FCR3S1.2 was mediated by a non-group A *Pf*EMP1 variant, FCR3S1.2*var*1 (also referred to as IT*var*21) by binding to heparan sulphate-like molecules. However, using semi-quantitative and quantitative (qPCR) methods, a recent follow-up study on the same parasite showed that rosetting was mediated by FCR3S1.2*var*2 (also known as IT*var*60) (Albrecht, Moll et al. 2011). IT*var*60, which is a group A *var* gene, was consistently shown to emerge as the dominant transcript immediately after cloning of FCR3S1.2 and even after 100 generations. IT*var*60 was also identified as the rosette-mediating variant in the same parasite line (also called IT/PAR+) by Ghumra *et al.*, 2012 (Ghumra, Semblat et al. 2012). It is not clear which receptors on uninfected red cells are involved in mediating rosetting in FCR3S1.2. However, the infected erythrocytes have been shown to bind soluble heparin, non-immune IgM and to form larger rosettes with blood group A red cells compared to O or B cells (Albrecht, Moll et al. 2011).

Var O is the third group A rosette-mediating *Pf*EMP1 variant that has been extensively characterized (Vigan-Womas, Guillotte et al. 2008; Juillerat, Igonet et al. 2010; Vigan-Womas, Lokossou et al. 2010; Juillerat, Lewit-Bentley et al. 2011). It is expressed by Palo Alto 89F5, a clone that was originally used in an *in vivo* experimental model in *Saimiri sciureus* monkey but later cultured in human RBCs (Vigan-Womas, Guillotte et al. 2008). *Var* O rosetting parasites dominantly expressed this variant that was absent in isogenic parasites expressing non-rosetting *var* R (Fandeur, Le Scanf et al. 1995; Contamin, Behr et al. 2000). Full-length *Pf*EMP1 (7.3kb) was identified by reverse transcriptase RCR (RT-PCR), chromosomal walking and sequencing using gene specific as well as degenerate primers (Kraemer, Gupta et al. 2003; Vigan-Womas, Guillotte et al. 2008).

In all these three variants described above, the N-terminal DBLα domain was shown to be the functional region that binds red cells to mediate rosetting (Rowe, Moulds et al. 1997; Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011). Four other rosette-mediating variants (shown in Table 4.1) were recently identified by Ghumra *et al., 2012* (Ghumra, Semblat et al. 2012). In each case, the *var* gene identified as encoding the probable rosette-mediating variant was the predominantly transcribed gene in rosetting (R+) parasites and was not transcribed, or transcribed at low frequency, in isogenic non-rosetting (R-) parasites. In three cases (HB3*var*6, TM284*var*1 and Muz12*var*1) the rosetting function was confirmed by the ability of specific antibodies to the variant to inhibit rosetting (Ghumra, Semblat et al. 2012). However, antibodies to the NTS-DBLα domain of TM180*var*1 did not inhibit TM180R+ rosetting, suggesting either that this variant does not mediate rosetting, or that domains other than NTD-DBLα are involved. TM180*var*1 should therefore be considered a "possible" rosetting variant,

which requires further study. PF13_0003 is another UpsA *var* gene found to have orthologous adhesion domain in 3D7 (Gardner, Hall et al. 2002). Parasites expressing this gene were shown to be rosetting (Vigan-Womas, Guillotte et al. 2011) and antibodies to NTS-DBLα domain had the ability to disrupt rosettes in homologous parasites.

Table 4.1: *Rosette-mediating PfEMP1 variants, including the name of the parasite, its geographical origin and GeneBank accession number for the var gene. TM180var1* is characterized as a possible rosetting gene in TM180R+ parasites.*

Rosetting Parasite	Origin	Rosetting *Pf*EMP1 variant	GeneBank accession Number
HB3R+	Honduras	HB3*var*6	PFHG_02274.1.
TM284R+	Thailand	TM284*var*1	JQ684046
IT/PAR+	South East Asia	IT*var*60	EF158099
Muz12R+	Papua New Guinea	Muz12*var*1	JQ684048
TM180R+*	Thailand	TM180*var*1	JQ684047
R29R+	South East Asia	IT*var*9	Y13402
Palo Alto Clone 89F	South East Asia	*Var*O	EU9082205
3D7	From strain NF54. Thought to be from Africa	PF13-0003	XP_001349740

Although epidemiological studies in Sub-Saharan Africa have shown an association between rosetting and severe disease (Carlson, Helmby et al. 1990; Treutiger, Hedlund et al. 1992; Rowe, Obeiro et al. 1995; Newbold, Warn et al. 1997; Kun, Schmidt-Ott et al. 1998; Heddini, Pettersson et al. 2001), most of the characterized *Pf*EMP1 variants described above are from isolates of non-African origin that have been in culture for a long time. Data from African field isolates

have shown distinct sub-groups of *var* genes being implicated in rosetting in natural parasite populations (Bull, Berriman et al. 2005; Kyriacou, Stone et al. 2006; Bull, Buckee et al. 2008; Warimwe, Fegan et al. 2012), hence it is possible to isolate and characterize rosette mediating *Pf*EMP1 variants from field isolates.

The field isolate described in this chapter is called SA075. It was obtained from a patient with severe malarial anemia from Kisumu, a malaria endemic area in western Kenya. The predominant gene expressed in 17/37 sequence tags in SA075 parasites grown in culture and selected for rosetting had previously been characterized by DBLα tag analysis (Bull, Buckee et al. 2008) as described in Chapter 2, section 2.3.9.3. Interestingly, the dominant tag sequence in SA075 was identical to a sequence isolated in Kilifi (EMBL Accession No. CAJ40433.1), from a rosetting isolate 4180 that had a rosetting frequency of 46% (Bull, Buckee et al. 2008). The sequence belonged to two *var* gene sequence groupings: -block-sharing group 2 and CP2, both of which have been associated with rosetting (Bull, Berriman et al. 2005; Bull, Buckee et al. 2008). The tag sequences were also grouped into sequence signatures also known as "sig", which consisted of a string of amino acids at each of the PoLVs together with the cysteine count (Bull, Berriman et al. 2005). SA075 contained sig2-like sequence features similar to what was seen in two highly rosetting field isolates from Kilifi (Bull, Berriman et al. 2005). Therefore, because of the suggested association of the SA075 tag with rosetting, there was need to identify and sequence the full-length *Pf*EMP1 variant from SA075 parasites. Following identification of the putative gene, recombinant proteins were made and antibodies raised from the different domains of the *Pf*EMP1 variant. Chapters 5 and 6 will focus on functional and immunological characterization of these anti-*Pf*EMP1 antibodies.

4.2: CHAPTER AIMS

The specific aims of this chapter were: -

1. To clone a recently culture-adapted field isolate, SA075 (Bull, Buckee et al. 2008), by limiting dilution.
2. To identify the dominantly expressed *var* gene transcript in SA075 rosetting clone.
3. To clone and sequence the full-length expressed *var* gene encoding the putative rosette-mediating *Pf*EMP1 variant from SA075.
4. To generate individual recombinant protein domains from the *Pf*EMP1 variant.
5. To raise antibodies to each recombinant domain to be used for functional assays as will be discussed in chapters 5 and 6.

4.3 MATERIALS AND METHODS

4.3.1 Parasite culture

The parasite isolate used in this study was called SA075 (details described in section 4.2). The parasite, a kind gift from Jose Stoute, was highly rosetting ex-vivo (Thathy, personal communication). Routine parasite culture maintenance and rosette selections for SA075 parasites were done as described in Chapter 2 sections 2.3.1 and 2.3.4 respectively.

4.3.2 Cloning of SA075 parasites by limiting dilution

Cloning of SA075 parasites was done by limiting dilution as described by Francois *et al.*,1994 (Francois, Hendrix et al. 1994), in which the culture suspension was first diluted to a pre-calculated probability of obtaining a given number of parasites per µl prior to inoculating in a flat bottomed 96-well tissue culture plate (Corning®

Costar®, Sigma Aldrich). The target dilution for cloning of SA075 was 0.4 parasites per well. A day before the actual dilution, parasites were synchronized to ring stage by Sorbitol method as described in Chapter 2, section 2.3.1. The next day at trophozoite stage, parasitaemia was determined by Giemsa stained thin smears (Chapter 2, section 2.3.1). The actual RBC count in the 20 ml culture suspension was then determined by a Coulter counter (Ac. T 5 diff CP, Beckman Coulter Inc. Miami Frorida USA). The culture at 5% parasitaemia and 2% haematocrit was then diluted in complete media down to 40 parasites in the 20ml suspension. 200µl of the diluted suspension was dispensed into each well to give a final parasite density of approximately 0.4 parasites/well. The outer wells around the plate were not inoculated with the culture suspension but were filled with culture media due to high rate of evaporation that occurred at the edges. Each cultured well was given an identification clone number after which the plate was transferred into a chamber, gassed for 1 minute and incubated at 37°C. Complete culture media was changed every 2 days and fresh, non-infected cells at 1% hematocrit added after every 5 days. Giemsa-stained smears were made from each well every week to determine presence of growing parasites. Once detected (after about 3 weeks), the parasite positive cultures were transferred to 25cc culture flasks. The percentage rosetting frequency (% RF) was thereafter determined for all the clones and two of them with the highest and lowest %RF were identified for reverse transcriptase PCR (RT-PCR). Agglutination assays as described in section 4.3.3 were also carried out on all the clones as an additional phenotype assay. Due to the nature of results of transcript profiles as will be discussed later, there was need to re-clone one of the highly rosetting positive clones (Clone 17). The process of re-cloning was similar to the one described above.

4.3.3: Antibody-mediated agglutination assays

Agglutination assay was done as previously described by Bull *et al.*, 2000 (Bull, Kortok et al. 2000). Trophozoite stage culture suspension from each of the clones was adjusted to 4% haematocrit in 1X PBS containing 0.5% BSA and 10μg/ml ethidium bromide. 11.5μl of the suspension was then incubated with 1μl of the hyperimmune serum called A1 in 96-well U-bottomed plates (Falcon, Becton Dickinson, USA) for 1 hour on a vertical rotor at room temperature. An unblinded negative control i.e. European serum on each of the clones was used as a reference while the assays were scored. Following the incubation, the entire assay volume for each well was transferred on to the corresponding pre-labeled microscope slide using a P20 pipette. A cover slip sealed with petroleum jelly was overlaid and the agglutinates counts were determined by fluorescence microscopy at X40 (Nikon eclipse 80*i*, Japan). An agglutinate was defined as a clump of 5 or more intact trophozoite-infected erythrocytes.

4.3.4 Var gene transcriptional profiling, cloning and sequencing of the full-length PfEMP1 variant

Following cloning by limiting dilution, *var* gene transcriptional profiling was done on the clone with the highest rosetting frequency (R+) and a non-rosetting (R-) clone as described in Chapter 2 section 2.3.9. The dominantly expressed *var* gene in R+ was identified for full-length cloning and sequencing by PCR walking strategy. This process involved PCR amplification from a known region of the DNA, in this case the DBLα tag region, to an unknown region upstream and downstream of the gene using variant specific and degenerate primers. The strategy was previously described by Kraemer *et al.*, 2003 (Kraemer, Gupta et al. 2003) and was used to amplify full-length *var* genes. Variant specific primers (listed in Table 4.2) were

designed within the SA075 sequence and paired up with the degenerate primers both upstream and downstream of the gene, a strategy illustrated in Figure 4.1. The variant specific primers within the tag were designed in the hypervariable regions so that they would be unique for the SA075 variant.

The first set of PCRs for the amplification of full length gene from genomic DNA involved Ups A, B and C forward primers (Table 4.2) that were paired up with a tag specific reverse primer in three separate PCR reactions (PCR 1 in Figure 4.1). Next, tag specific forward primer was paired up with reverse degenerate primers (Kraemer, Gupta et al. 2003) from the downstream domains i.e. DBLβ, DBLγ, DBLδ and Exon II in four separate PCR reactions (PCR 2 in Figure 4.1). A product from any of these amplification reactions was then cloned into a vector (Chapter 2 section 2.3.9.3) and 10 colonies randomly picked for sequencing. The new sequences would then be assembled with the already known tag sequence and new primers designed for subsequent PCRs further upstream and downstream (PCR 3,4, and 5 in Figure 4.1). To ensure the correct gene had been amplified, the new sequence was to overlap with the known tag sequence at 100% nucleotide identity. Sequence assembly was done using Seqman Pro, Lasergene. All sequencing work described in this section was done at the GenePool facility at the University of Edinburgh. A list of all variant specific and degenerate primers that were used in sequencing of the full-length SA075 gene is shown in Table 4.2.

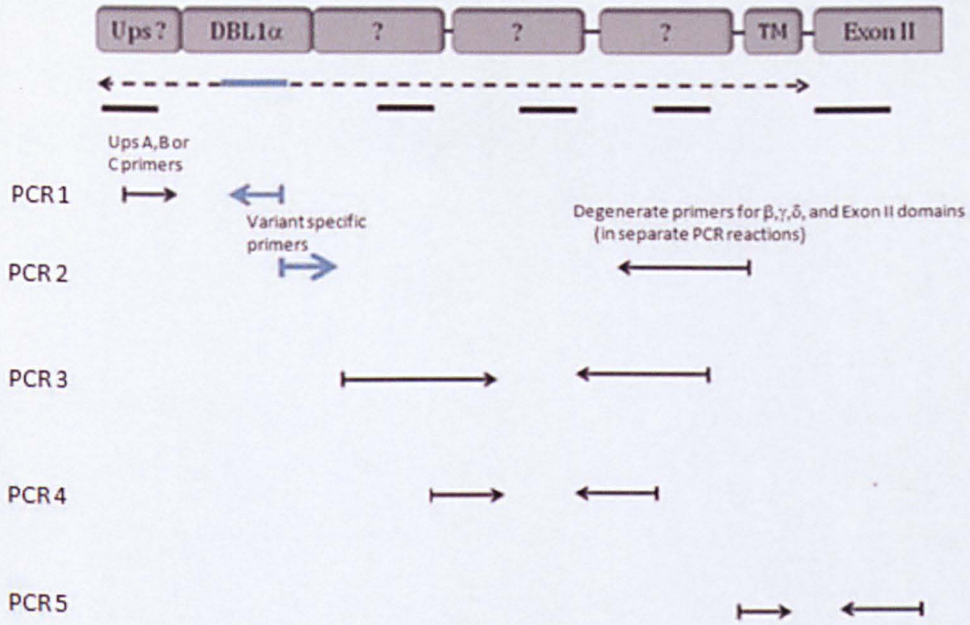


Figure 4.1: *Schematic representation of the PCR strategy used for sequencing of the full-length PfEMP1 variant in SA075 starting from the known DBLα tag region (represented in blue) to unknown regions upstream and downstream of the gene (in dotted line). Tag specific primers (in blue) were first paired up with Ups primers and degenerate primers (in black) upstream or downstream of the gene. The first set of PCR reactions (PCR 1) was done using forward primers for Ups A, B or C paired up with a variant specific reverse primer of the DBLα tag region, in three separate reactions. The reaction involving the Ups B primer yielded a product of approximately 1.5kb which was then cloned and sequenced. The second set PCR reactions (PCR 2) involved a variant specific forward primer paired up with degenerate primers from each of the downstream domains (β, γ, δ and Exon II) in separate reactions. While other domain primers did not have any product in the first PCR attempt, the PCR involving DBLγ primer yielded a product (~ 6kb) that was also cloned and sequenced. Since the product from PCR 2 was large, subsequent PCRs 3,4 were done using variant specific primers designed from the*

already sequenced region that were used to amplify and sequence the region between the tag region and the gamma domain. Finally a PCR was done (PCR 5) using a forward primer designed at end of DBLγ domain and paired up with exon II reverse primer and the product sequenced and assembled. The full-length gene was finally sequenced and named SA075var1.

Table 4.2: *Sequences for variant specific and degenerate primers used for cloning and sequencing of full-length gene SA075. The first 7 primers are degenerate primers from Kraemer et al., 2003 (Kraemer, Gupta et al. 2003). Primers 8-23 were variant specific designed from SA075 gene.*

	Primer name	Sequence 5'-3'
1	Ups A	AAT ATW ATG AAT CTT CAG
2	Ups B	TTGCCTCTDTTGTTATCTC
3	Ups C	GTGATAACYAYTATATMATATAC
4	DBLβ_3.2	AATC(G/T)TTG(A/G/T)GG(A/G)AT(A/G)TA(A/G)TC
5	DBLγ_3.2	ACCATC(G/T)(C/T)A(A/G)(A/G)AA(C/T)(G/T)G(A/G)GG
6	DBLδ_3.1	G CCCCATTC(A/T)T(C/G)(A/T)A(A/G)(C/G)(C/T)A(A/T)CG
7	Exon_2 3.3	CCADYTCTTCATAYTCACTTTC
8	SA075 Tag Forward	ACGAACTAAATAATCTTTCATTGGA
9	SA075 Tag Reverse	CCATCTGTAAATTCCCGTTTC
10	SA075 gamma Fow 1(Towards exon 2)	CATCATATTAGAGACACCTCG
11	SA075 gamma Rev 1 (Towards internal)	ATTCCTCTTATCACACTCCCACGC
12	SA075 gamma Rev 2 (Towards internal)	GCTGCAGATTTGATGAATGCT
13	SA075 alpha/gamma Fow 1 (400bp from end of tag)	TTACTAACAGTGCTGATGACA
14	SA075 alpha/gamma Fow 2 (200bp from end of tag)	TTCTAGGGATAGTAAGTGTACTGC
15	SA075 alpha _gamma internal 2 FOW	GCATAGATTATACTAACGTCACC
16	SA075 DBL alpha _gamma internal 2 REV	ATCTACCACTTGTTGATAGTCG
17	SA075 DBL gamma_delta internal 2 FOW	CTCCAGGTAATGTTGATGTAC
18	SA075 DBL gamma_delta internal 2 REV	CTAGATGATTTTCTGCTGCCTG
19	SA075 Delta (outward) FOW	CGAGACAGTCAAACTCGAC
20	SA075 INT FOW (original)	TAGACACCATAAAATGGGAAACCA
21	SA075 INT REV	TTCATCGCTCGACATACAGTCTGC
22	SA075 Exon Fow	TGCAGCGATGCTAAAGGAAATACTC
23	SA075 Exon Rev	GCCTTTGTATTTATCAGTACCATAG

For all the PCRs, a 50 µl reaction was set up that consisted of 1X *Pfx* amplification buffer, 10mM dNTPs, 2.5mM Mg_2SO_4, all from Platinum® *Pfx* polymerase kit (Invitrogen), 0.25µM forward and reverse primers, 100ng DNA, 1U *Pfx Taq* polymerase all topped up to 50 µl with DNAse free water. The PCR machine used was a PCT-100 Peltier Thermal Cycler PCR machine (MJ Research) with the following conditions: -1 cycle of 94 °C for 5 minutes followed by 35 cycles of 94 °C for 5 seconds, *48 °C for 15 seconds, 60 °C for 2 minutes 30 seconds and a final extension at 60 °C for 10 minutes. The annealing temperature (marked in asterix) however varied for the different PCRs due to different melting temperature of primers (Table 4.3).

Table 4.3: *Annealing temperatures for the different PCR reactions used to amplify and sequence full-length SA075 gene*

PCR reaction	Primer sequence	Annealing temperature (°C)
PCR 1	**Ups B:** TTGCCTCTDTTGTTATCTC **SA075 Tag reverse** CCATCTGTAAATTCCCGTTTC	45
PCR 2	**SA075 Tag Forward** ACGAACTAAATAATCTTTCATTGGA **DBLγ_3.2** ACCATC(G/T)(C/T)A(A/G)(A/G)AA(C/T)(G/T)G(A/G)GG	56
PCR 4	**SA075 DBL gamma_delta internal 2 FOW** CTCCAGGTAATGTTGATGTAC **Exon2 3.3** CCADYTCTTCATAYTCACTTTC	48
PCR 5	**SA075 Delta (outward) FOW** CGAGACAGTCAAACTCGAC **SA075 Exon Rev** GCCTTTGTATTTATCAGTACCATAG	50

Although Platinum *Pfx* offers a higher fidelity than the ordinary *Taq* polymerase enzymes, it does not add an A overhang naturally and so the PCR products generated by *Pfx* PCR above required further treatment prior to TA cloning. To do this, the products were first purified using the QIAquick PCR Purification kit (Qiagen 280006). 5 volumes of PB buffer containing guanidine hydrochloride and isopropanol was added to the products in a column and spun down. All centrifugations for this protocol were at 13000rpm for 1 minute in a bench-top centrifuge. 750 µl wash buffer PE was added to the column and spun again. The flow-through was discarded and the column spun one more time to remove any residual ethanol. Products were then collected in a fresh tube after addition of 30 µl elution buffer (10mM Tris-HCl, PH 8.5) to the column and a final spin. The purified product was then incubated with 1U *Taq* polymerase (Invitrogen) at 72°C on a heating block in readiness for the ligation and sequencing as described in Chapter 2, section 2.3.9.3.

4.3.4.1: Amplification and digestion of PfEMP1 domain constructs for protein expression

Once the putative full-length *var* gene associated with rosetting in SA075 was identified, recombinant proteins were made from the different domains. The first step in protein expression involved amplification and digestion of the different domains of the *Pf*EMP1 variant. The primers and restriction enzymes used for each domain are shown in Table 4.4. Domain boundaries for the constructs were retrieved from the *Var*Dom 1.0 Server (http://www.cbs.dtu.dk/services/VarDom/), a software that predicts and classifies *Pf*EMP1 domains and homology blocks based on analysis from 7 genomes (Rask, Hansen et al. 2010). In certain instances however, more than one construct was made per domain to improve on the

solubility of the protein. In such cases, the C-terminal of the DBL domain would be extended or reduced by 1 or 2 cysteine residues.

Table 4.4: *Primer sequences and domain boundaries for the SA075var1 constructs. Also shown are restriction enzymes and conditions for restriction digest including the NEB buffer used, incubation temperature and presence or absence of Bovine Serum Albumin (BSA).*

Domain Construct	Domain (amino acid) boundaries	Forward and reverse primers 5'-3' sequence (Restriction enzyme)	Conditions for restriction digestion (50µl reaction)
NTS-DBL1α	1-474	AAGGATCCATGGCGCGACGTC GTCGTGGT (BamHI) AACTCGAGTTAGGGACACACTT GGCAATATTC (Xho)	25 µl of product in 5 µl NEBuffer 3, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours
NTS-DBL1α-CIDR1α (Didomain)	1-726	AAGGATCCATGGCGCGACGTC GTCGTGGT (BamHI) AACTCGAGTTAACATGCTTCGTT TGAATTATTGTC (XhoI)	25 µl of product in 5 µl NEBuffer 3, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours
DBL2β	735-1186	AACTCGAGCCCTGTGGAAGAAA CAATAATGGT (XhoI) AACATATGTTATCCATCCACTAT TTCACACGCATC (NdeI)	25 µl of product in 5 µl NEBuffer 4, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours
DBL3γ	1187-1535	AAGGATCCATACTTAATGGAAA GAGCGCAACGA (BamHI) AAGCTAGCTTATGCGGGCATAT CAACTTCAGTA (NheI)	25 µl of product in 5 µl NEBuffer 2, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours
DBL4δ	1697-2187	AAGGATCCCCATGTGAAATAGT ACAAAAACTATTTAC (BamHI) AAGCTAGCTTAAACATGGTTTAC AATCTTCTGC (NheI)	25 µl of product in 5 µl NEBuffer 3, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours
CIDR1α	475-726	AAGGATCCGATTGTGTAGTTGA ATGTGTCGA (BamHI) AAGCTAGCTTAACATGCTTCGTT TGAATTATTGTC (NheI)	25 µl of product in 5 µl NEBuffer 4, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours
CIDR2β	2188-2439	AAGCTAGCTCTTCATTTAAAATA GATTGTG (NheI) AACTCGAGTTAACACGCTTGGT TTTGG (XhoI)	25 µl of product in 5 µl NEBuffer 4, 0.5 µl 1X BSA and 1.5 µl of enzymes incubated at 37°C for 2 hours

Expression constructs were made for each of the four DBL and two CIDR domains, as well as a di-domain representing the *Pf*EMP1 head structure (NTS-DBLα-CIDRα). Primers with restriction enzyme sites incorporated at the ends were designed for each construct to allow for cloning into the expression vector. The reverse primers contained a stop codon (5' TTA 3') that would end protein expression for the construct. Restriction sites within the insert sequences were checked using the NEB cutter v2.0 software (New England Biolabs. Inc) to ensure that enzymes used to make the primers did not have restriction sites within the insert. Table 4.4 shows primer sequences and restriction enzymes that were used for the different constructs.

Genomic DNA (gDNA) from SA075 was amplified using the above primer pairs in a 50 µl PCR reaction that was done using the Platinum® *Pfx* polymerase kit (Invitrogen). The reaction contained 1X *Pfx* amplification buffer, 10mM dNTPs, 2.5mM Mg_2SO_4, 0.5µM forward and reverse primers, 100ng DNA, 1U *Pfx* polymerase, topped up to 50µl with DNAse free water. Apart from the annealing temperature, cycling conditions were similar for all the constructs (Table 4.5).

Table 4.5: *Final cycling conditions for amplification of the different SA075var1 constructs. The annealing temperatures for the different constructs were as follows: - NTS-DBLα: 68°C, Di-domain: 66°C, CIDRα: 66°C, DBLβ: 64°C, DBLγ: 68°C, DBLδ:65°C and CIDRβ: 60°C.*

Amplification step	Cycling conditions
Initial denaturation	94°C for 5 minutes
Denaturation Annealing Extension	94°C for 30 seconds Varied for different constructs 60°C for 3 minutes } X 35
Final extension	60°C for 10 minutes

5µl of the above PCR products were run on a 1.5 % agarose gel (Chapter 2 section 2.3.9.4.) alongside a 1kb DNA ladder to check for presence of a band. The remaining 45µl of PCR product was purified using QIAquick PCR purification kit (Qiagen) as described in section 4.3.4 in which the final elution of the purified product was done with 30µl of elution buffer. A restriction digest of the product was then done on 25µl of the product using respective enzymes for each construct (Table 4.4) to allow for cloning into the expression vector. Following the digestion reaction, 5 µl of the product was run on a 1% agarose gel to check for the efficiency of digestion while the remaining 45µl was loaded far away from the first well to avoid exposure to UV. Using a ruler, the position of the band on the non-exposed band was determined using the exposed piece of gel and was carefully excised out of the gel. The product was gel purified using QiaQuick gel extraction kit (Qiagen 28704). This was done by first weighing the piece of gel and adding QG buffer to it in an Eppendorf tube at a volume three times the weight of the gel. This was heated at 50°C for 10 minutes before 1 gel volume of isopropanol was

added. The rest of the purification process on a spin column was carried out as described in section 4.3.4 above.

4.3.4.2: Preparation of expression vector_modified pET15b

The expression vector used was pET15b that was modified by Matt Higgins (Higgins 2008). The modification was done by insertion of DNA encoding an N-terminal hexahistidine tag and a cleavage site for the Tobacco Etch Virus (TEV) protease. Unlike thrombin which is used as the protease cleavage site in the original pET15b vector (Novagen), TEV protease is highly site-specific, highly active and was easily produced and purified in the lab in large quantities. The modified vector also contained an additional cloning site (NheI) located between BamHI and XhoI in frame with the TEV cleavage site. Figure 4.2 shows the cloning/expression region of pET15b showing regions that were modified.

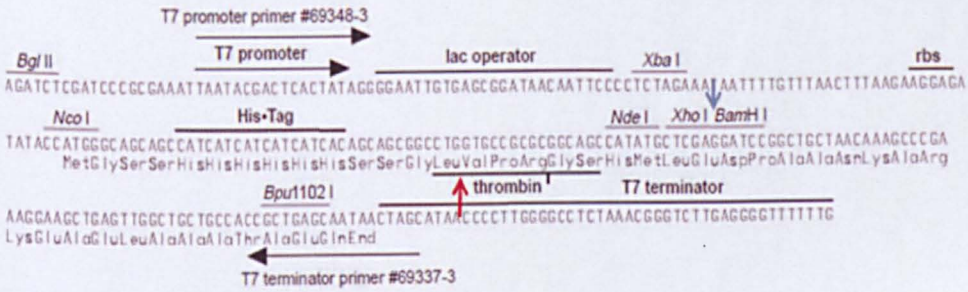


Figure 4.2: *Map of cloning/expression region of the original pET-15b vector by Novagen which was modified by Matt Higgins by the addition of an extra cloning site (NheI) in between BahmHI and XhoI (blue arrow) and replacement thrombin with TEV as a protease cleavage site (red arrow) (Higgins 2008).*

4.3.4.3: Cloning of purified SA075 PfEMP1 constructs into the prepared pET15b vector

The purified and digested PCR products from each of the constructs described in section 4.3.4.1 were ligated into restriction enzyme-digested and shrimp alkaline phosphatase (SAP)-treated pET15b vector. SAP treatment was done to prevent self-ligation of the vector. The SAP reaction (Promega) was done on 30 µl of digested vector using 3.5 µl SAP 10x buffer (0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 8.5) and 2 µl SAP enzyme. The mixture was incubated for 15 minutes at 37°C followed by a 15 minute inactivation of the enzyme at 65°C.

A ligation reaction was then done using a T4 DNA ligase kit (Invitrogen, UK) followed by an overnight incubation at 16°C. The ligation reaction consisted of 7µl of the construct product in 1 µl of the SAP-treated vector using 1 µl T4 DNA ligase enzyme in 1 µl of 10X ligation buffer (500mM Tris-HCl, 100mM $MgCl_2$, 10mM ATP, 100mM Dithiothreitol, pH 7.5). The following day, the ligated plasmid was used to transform One Shot® TOP10F' chemically competent *E.coli* cells (Invitrogen™). The competent cells were first thawed-out in ice and 2µl of plasmid gently added and mixed. After a 30 minute incubation in ice, the cells were heat shocked for 30 seconds at 42°C in a heating block and put back in ice for 2 minutes. 250 µl of SOC media (Invitrogen) was added to the transformed cells and placed on a shaking incubator for 1 hour at 37°C. The cells were then plated onto Luria Bertani (LB) agar (Sigma) plates containing 50µg/ml of ampicillin (Sigma-Aldrich) as described in Chapter 2, section 2.3.9.3. The agar plates were supplemented with 40 µl of 20µg/ml IPTG and 20µg/ml X-gal for blue-white screening. 10 random white colonies were then picked and grown overnight in LB broth (Sigma-Aldrich) supplemented with 50µg/ml ampicillin at 37°C in a shaking incubator at 200rpm.

Plasmid DNA was extracted using a modified QIAprep Spin miniprep kit (Qiagen) protocol. In the modified miniprep protocol, the spin columns provided in the kit were avoided since the pET15b vector got stuck onto the columns resulting in reduced amounts of plasmid DNA (Ghumra, personal communication). 1 ml of bacterial cultures from individual colonies was first centrifuged at 13000 rpm for 3 minutes. The pellet was then resuspended in 250 µl buffer P1 (6.06g/l Tris base, 3.72 g/l EDTA, pH 8.0) before lysis with 250 µl of P2 buffer (8g/l NaOH, 1% (w/v) SDS in water) and subsequent neutralization with 350 µl N3 buffer (confidential composition). Cell debris were removed by centrifugation at 13000rpm for 10 minutes. According to the modified protocol, the supernatant was transferred into a fresh 1.5 ml Eppendorf tube instead of the columns and spun down at 13,000rpm for 8 minutes to remove any precipitates. This was then transferred to another fresh Eppendorf tube and 630 µl of Isopropanol (Sigma) was added followed by vortexing and spinning down for another 8 minutes at 13,000rpm. A white pellet of DNA formed at the bottom of the tube and the supernatant was carefully poured out. This was followed by a wash with 200 µl of 70% Ethanol (Sigma). The pellet was air dried for 5 minutes and then resuspended in 50 µl of clean water. To check for the presence of inserts in the plasmids, a diagnostic digest was done on each of the 10 plasmids using enzymes and conditions described on Table 4.4. Plasmids that were confirmed to contain the insert were then prepared for sequencing to check that the insert was in the right frame and did not contain any PCR-generated mutations. The big dye reaction consisted of 7 µl of the plasmid, 1 µl of big dye and 1 µl of T7 promoter primer (TAA TAC GAC TCA CTA TAG G). The reaction mix was incubated for 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Sequencing was done at the GenePool facility at the University of Edinburgh.

4.3.4.4: Pilot protein expression

Once the plasmid was confirmed to contain the *Pf*EMP1 construct in the correct frames and without any PCR-generated mutations, a pilot protein expression (usually done in a small volume of 6 mls) was carried out for each of the constructs to optimize conditions for maximum expression. This was also done to determine whether the proteins were soluble or not. Once optimum conditions were obtained, a large scale expression of up to 4-8 litres would then carried out. The process was carried out as described by Ghumra *et al.,* 2011 (Ghumra, Khunrae et al. 2011). Plasmids that had no mutations and which were confirmed to be in the correct reading frame were transformed in Origami B *E.coli* cells (Novagen, Nottingham, UK) (Baca and Hol 2000) by adding 2 μl of the plasmid into 50 μl of the cells which were also already transformed with pRIG plasmids. The transformation process was done as described in section 4.3.4.3 above. The pRIG plasmids are important for expression of genes with biased codons such as the AT-rich *Plasmodium falciparum* (Baca and Hol 2000). They contain genes coding for three tRNAs:- arginine, isoleucine and glycine, that recognize rare codons hence increasing the level of expression of the *P.falciparum* proteins in *E.coli.*

The transformed Origami B cells were then plated onto 2XYT (Sigma-Aldrich) agar plates containing 50μg/ml Ampicillin. The agar plates were made by adding 35g of 2XYT agar powder (Sigma) into one litre of water followed by autoclaving. On cooling down, Ampicillin was added to a final concentration of 50μg/ml and the solution poured out onto 100mm petri dishes for cooling. 50μl of the bacteria was plated and allowed to grow overnight at 37°C. The following day 10-20 colonies were picked and placed into 6mls of sterile 2XYT broth made by dissolving 31g of

the powder per litre of water before adding 50μg/ml Ampicillin. This was grown overnight in a 37°C shaking incubator at 175 rpm. Following day, 0.6mls of overnight culture was inoculated into 6mls (1:10 dilution) of 2XYT broth and grown in a 37°C shaking incubator at 175 rpm. The optical density (OD) measured at 600nm was constantly monitored using a spectrophotometer (Biophotometer-Eppendorf) in a 1ml disposable cuvette. A blank sample (2XYT media) was first measured at 600 O.D after which the cuvette was rinsed with water and 200 μl of growing bacterial culture was measured. Once the OD reached between 0.6-0.8, a 1 ml glycerol stock was made by adding 150 μl of glycerol (Sigma) to 850 μl of the bacterial culture and storing at -80°C. The stock would be used for inoculating the large scale protein production, in case the protein was soluble. If not, the glycerol stock was discarded.

The remaining approximately 5mls of culture from the pilot expression was allowed to grow up to an OD of about 1.2 during which IPTG induction was done. However, just before induction, 1ml of culture was aliquoted in an Eppendorf tube, centrifuged for 1 minute at 13000rpm and the pellet resuspended in 1X PBS and frozen down as the "uninduced control". The remaining culture was induced with IPTG whose concentration varied for the different constructs (Table 4.6). This was allowed to grow for 3 hours at 20°C and 175rpm after which a second 1 ml aliquot was taken and labeled "3 hours after induction". The remaining culture was left to grow overnight in a shaking incubator at 175rpm and the following morning the final pellet labeled "overnight induction" was obtained and frozen as above. The specific length of time and temperature for induction were optimized for each construct as shown in Table 4.6

Table 4.6: *Optimized expression conditions for the different SA075 constructs including IPTG concentration, induction temperature and length of induction.*

Name of the construct	IPTG concentration	Induction temperature (°C)	Length of induction
NTS-DBL1α	0.5mM	20	Overnight
NTS-DBL1α-CIDR1α	0.25mM	16	36 hours
CIDR1α	0.3mM	16	24 hours
DBL2β	0.3mM	16	Overnight
DBL3γ	0.25mM	16	24 hours
DBL4δ	0.5mM	16	24 hours
CIDR2β	0.25mM	16	24 hours

To check for the presence of the protein and whether it was soluble or insoluble, the 3 pellets i.e. 1) uninduced, 2) 3 hours after induction and 3) overnight induced, were all thawed out at room temperature and sonicated (Branson Sonifier 450 Sonicator) by pulsating three times for 5 seconds with gaps of 5 seconds. 20μl was taken from each tube as the total fraction, mixed with 5μl of Invitrogen SDS-PAGE loading buffer and reduced using 1μl of β-mercaptoethanol (Sigma). The reduction reaction was done by incubating at 75°C for 15 minutes. The remaining sonicated lysate was spun down for 1 minute at 13,000rpm in a microfuge and 20μl suspension taken as the soluble fraction. Each pair of the soluble and total fraction was then run side by side on precast NuPage Novex Bis-Tris Agarose gels

(Invitrogen). The 4-12% gels were made of Bis-Tris-HCl buffer (pH 6.4), Acrylamide, Bis-acrylamide, Ammonium Persulfate (APS) and Ultrapure water. The 12 well gel cassette was then placed into a XCell *SureLock*™ Mini-Cell (Invitrogen) and 800 mls of 1X MOPS running buffer (3-(N-morpholino) propane sulfonic acid) was added. 15µl of the soluble and total fraction samples were loaded alongside the Precision Plus Prestained Standard molecular weight marker (Bio-Rad). After electrophoresis, the proteins were transferred onto PVDF membrane (Thermo Scientific) in a western blot and the protein detected using an HRP-conjugated anti-6X-His tag antibody (Qiagen, Crawley, UK) at a dilution of 1:5000. The western blot was done by first carefully placing the gels onto PVDF membrane that was pre-soaked in 1X NuPage transfer buffer, supplemented with 20% methanol. Since the samples were reduced, 0.1% NuPAGE® Antioxidant was added into the transfer buffer to keep the proteins in a reduced state and prevent re-oxidation. The membrane/gel was then carefully placed in between pre-soaked filter papers and blotting pads which were also soaked in the transfer buffer. The whole assembly was then carefully locked onto an XCell II™ Blot Module which was then filled up with transfer buffer and run at 30volts for 1 hour.

After the 1 hour run, the membrane was then rinsed in wash buffer (1X PBS with 0.1% Tween-20) and blocked for 2 hours with 5 % milk in 1XPBS/0.1%Tween-20. This was followed by two more washes after which detection antibody, mouse anti-rabbit conjugated with HRP was added at 1:5000 in wash buffer. This was incubated for 1 hour at room temperature while gently shaking. After rinsing twice with 1X PBS, a substrate 1:20 dilution of DAB (3'3 diaminobenzidine tetrahydrochloride) (Thermoscientific) in 1XPBS was added which reacts with HRP in the presence of 1:1000 dilution of hydrogen peroxide to produce a brown coloured band.

Once soluble protein was detected by the western blot, a large scale of usually 4-8 litres was prepared. The large culture was inoculated using the glycerol stock that was stored in -80°C during the pilot expression by taking 250 µl of the thawed out stock into 100mls of 2XYT media containing 50 µg/ml Ampicillin and grown overnight at 37°C in a shaking incubator at 175 rpm. The following day, 10 mls of the culture was used to inoculate each of the 1 litre flasks and grown at 37°C while monitoring the OD. The rest of the conditions for the large scale protein expression were done as in the pilot expression (Table 4.6).

A major modification from the original protein expression protocol by Ghumra *et al.*, 2011 (Ghumra, Khunrae et al. 2011) was the inclusion of a heat shock step, a strategy that was introduced prior to IPTG induction in order to improve the expression of soluble proteins (Oganesyan, Ankoudinova et al. 2007). Briefly, the bacterial culture was grown up to an OD value of between 0.6 and 1.2. Just before IPTG induction, the culture was incubated at 42°C for 10 minutes followed immediately by a 30 minute incubation on ice and another 20 minutes incubation at 37°C.IPTG induction and overnight growth were done as previously described (Table 4.6).

4.3.4.5: Purification of the recombinant proteins

Bacterial pellet from the 4-8 litre preparation was harvested in 600ml wide mouth bottles (Nalgene) by centrifugation at 4600 rpm (Heraeus, Thermo Fisher Scientific Inc.) for 30 minutes at 4°C. After the first spin, the supernatant was carefully decanted without dislodging the pellet. The remainder of the bacteria was added into the same bottle and spun as before until pellet from the whole 4-8 litre bacterial culture was collected. After the final spin, the pellet was re-suspended in

lysis buffer which contained 20mM Tris pH 8, 0.3M NaCl, 15mM Imidazole, 0.5% Triton-X and EDTA-free protease inhibitor tablet (Mini-complete, Roche). Pellet from 1 litre bacterial culture was re-suspended in 15 mls of lysis buffer in 50 ml Falcon tubes. Sonication (Branson Sonifier 450 Sonicator) was done five times at 15 microns power using a 9mm probe for 10 seconds, while keeping the sample tube cool in a beaker of ice. The lysate was then spun down at 20,000rpm for 20 minutes (Sorvall centrifuge, Thermo Scientific) in NALGENE® oak Ridge Tubes. Supernatant was carefully aspirated and passed through a pre-equilibrated Nickel Nitriloacetic Acid (Ni-NTA) sepharose matrix (Qiagen) in a Bio-rad Econo-chromatography column that had been equilibrated with lysis buffer. The Ni-NTA matrix allowed for the 6XHis-tagged protein to bind onto the agarose via the His-tag. It was always used fresh but could be stored at 4°C in 20% ethanol to be re-used for future purification of the same protein.

The bound protein was then washed in 400mls of lysis buffer and finally eluted using 10 mls of buffer containing 20mM Tris pH 8, 150mM NaCl and 200mM imidazole. Elution was repeated 5 times with each elute being collected separately and later quantified by Nanodrop. Eluates with high amounts (>0.1g/L) of protein were pooled together before protein buffer exchange was done using PD-10 Desalting columns (Bio-rad). Buffer exchange was done by first equilibrating the desalting columns with 10mls of 1X PBS. Protein in elution buffer was added to the columns (2.5 mls per column) before finally eluting the protein in 3.5 mls of 1XPBS. TEV protease cleavage was done overnight at room temperature using 3mM reduced glutathione, 0.3 mM oxidized glutathione buffer at an approximate ratio of 1 mg of protease to 10 mg of protein. The protease (usually stored in 1 mg/ml aliquots) was added to the protein in a 50 ml Falcon tube and left overnight at room temperature. The following day, the mixture was passed through a Ni-NTA

affinity column (same one used for purification) that had been equilibrated with 1X PBS to remove any uncleaved protein. Since the TEV protease is His-tagged, it was removed from the final protein during this step. The eluted protein was finally concentrated using Vivaspin 20 columns (Sartorius Stedim) by first equilibrating the columns with 1X PBS and spinning for 5 minutes at 4600rpm at 4°C. The flow through was discarded and the protein added and spun down for 30 minutes at 4°C. During the course of spinning, the concentration of the protein was constantly checked using a nanodrop. Once the volume reached 5 mls the protein was finally quantified using a nanodrop and frozen down in aliquots at -80°C, awaiting immunization for antibody generation. Some proteins however required further spinning down to 2-3 mls to allow for a more concentrated protein solution. The minimum quantity of protein required for antibody production (section 4.3.5) was 1.5 mg.

The final protein purity was checked by SDS-PAGE on 10% bis-Tris polyacrylamide gels stained with SimplyBlue™ (Invitrogen) as described in section 4.3.4.4. 2μg of proteins was reduced using 5% β- mecarptoethanol by heating to 75°C for 15 minutes and run in parallel with the non-reduced protein for comparison. The gel was run at 120 volts for 1.5 hours and the protein carefully removed from the cassette. Staining was done using SimplyBlue™ SafeStain (Invitrogen) in which the gel was placed in a container with 100ml of distilled water and boiled three times in a microwave for 1 minute until the solution was close to boiling. 20 mls of the SimplyBlue stain was then added and boiled in a microwave for 45 seconds. The dye was later washed off with 100mls of water on a shaker.

4.3.5: Generation of domain specific polyclonal antibodies in rabbits

Immunization and generation of antibodies was done by BioGenes GmBH (Berlin, Germany). The immunization schedule for the protein is summarized in Table 4.7. Prior to immunization, five rabbits were selected and their pre-immune sera tested for non-specific reactivity on infected and non-infected RBCs by IFA assay (Chapter 2, section 2.3.10) Two of the rabbits with the least/or no background were then selected for immunization which was carried out using an adjuvant that contained 0.23% of lipopolysaccharides of the blue-green algae *Phormidium* spp, 92.8% mineral oil, 3.48% Tween-20, 3.48% Span-80. On day 28, an IFA assay was done on live SA075 parasites using anti-sera from the two immunized rabbits. The rabbit antiserum that gave the brightest positive signal was chosen for a final boost after which the antiserum was obtained for protein-A purification of total IgG (Biogenes GmbH). IgG from a non-immunised rabbit was also purified by the same method to be used as a negative control. All polyclonal antibodies from all the recombinant domains as well as the anti-serum was stored at -20°C awaiting functional assays as will be discussed in Chapter 5.

Table 4.7: *Immunization schedule for rabbits used in the production of anti-PfEMP1 polyclonal antibodies. Rabbits were immunized with 250μg of protein on day 1 and 100μg on subsequent immunizations.*

Days	Procedure
Day 1	Pre-immune serum (1,5 ml per rabbit)/1st immunization
Day 7	2nd immunization
Day 14	3rd immunization
Day 28	Bleeding (25 ml per rabbit) / 4th immunization
Day 35	Bleeding (25 ml per rabbit)
Day 49	Final immunization
Day 56	Final bleed and purification of total IgG from one animal

4.4: RESULTS

4.4.1: Cloning of SA075 parasites by limiting dilution and transcriptional profiling of R+ and R- clones

Unlike in Chapter 3 where *var* gene transcriptional profiling in field isolates was done on gelatin-selected isogenic parasite pairs, this chapter describes transcriptional profiling on cloned parasites. Cloning by limiting dilution was done to obtain single cell derived populations of rosette positive (R+) and rosette negative (R-) parasites. From the 60 wells that were inoculated with culture suspension during the first round of cloning, 27 (45%) wells (Table 4.8) turned out to be parasite positive following Giemsa staining. These were tested for rosetting (described in 2.3.5) and agglutination (described in 4.3.3) out of which 13 (49%) were rosette positive while 2 (7%) formed medium sized agglutinates. The clone with the highest % RF, (44.9%) (Clone 17) and one with 0% (Clone 13) were picked for comparison of transcript profiles.

Table 4.8: *% RF and the agglutination scores for the 27 parasite positive clones obtained in the first round of cloning of SA075. Clone 13 and clone 17 were selected for DBLα tag analysis.*

Clone ID	%RF	Agglutination score
Clone 3	0.0	0
Clone 4	0.0	0
Clone 5	12.6	0
Clone 6	0.0	0
Clone 7	0.0	0
Clone 8	9.2	0
Clone 9	0.0	0
Clone 10	0.0	0
Clone 11	5.0	0
Clone 13	0.0	0
Clone 14	0.0	0
Clone 15	9.1	0
Clone 16	0.0	0

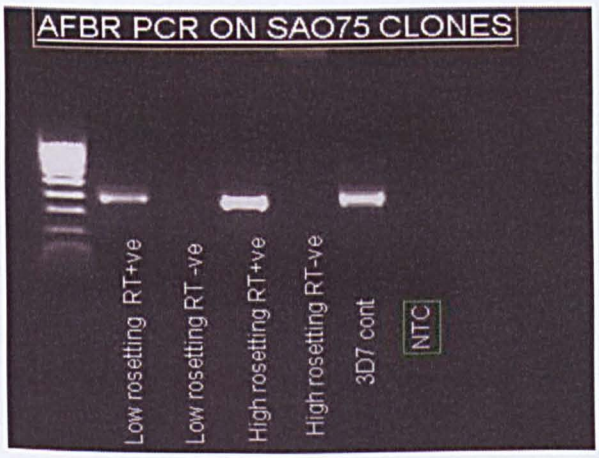
Clone 17	44.9	0
Clone 18	2.4	0
Clone 19	4.3	25
Clone 20	6.9	0
Clone 21	0.0	0
Clone 22	0.0	0
Clone 23	5.0	0
Clone 24	0.0	0
Clone 25	0.0	0
Clone 26	5.9	0
Clone 27	11.3	0
Clone 35	3.8	0
Clone 36	8.2	0
Clone 74	4.0	12

The DBLα tag was successfully amplified from cDNA preparations of clones 13 and 17, as previously described in Chapter 2, section 2.3.9. The PCR products were devoid of genomic DNA contamination as seen in reverse transcriptase negative (RT-ve) control lanes (Figure 4.3, Panel A).

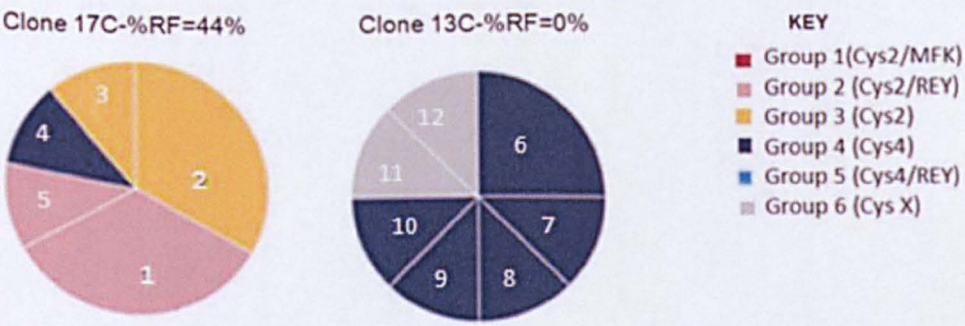
DBLα tag sequences from clones 13 and 17 were then analyzed (Chapter 2, section 2.3.9.5). Each unique transcript sequence was given an arbitrary identity as SA075_1,2,3 etc as shown in Figure 4.3, Panel C. The sequences were then classified based on the previously described CP classification algorithm (Bull, Berriman et al. 2005) with each of the 6 different CP groups being assigned a unique colour code (key of figure 4.3, Panel B). In the R+ clone, 18 sequences were obtained. Two dominant *var* gene transcripts: SA075_1 and SA075_2 came up, both of which had two cysteine residues. The two sequences belonged to CP2 and a CP3 respectively as shown in Figure 4.3, Panel B. Note that SA075_1 is the same tag sequence previously been identified in SA075 (described in chapter 1, section 1.8). Both sequences were in equal proportions (36%_6 sequences each) and therefore it was not possible to identify a single predominant gene as a

rosetting ligand candidate. Interestingly, both these genes were missing in the R- transcript profile which was composed mainly of Cys4 sequence types (Figure 4.3, Panel B). Despite not having a clear dominant gene in R+ clone, the overall distinction between cys2 and non-cys 2 in the R+ and R- clones respectively, was consistent with previous studies on the association of Group A or cys2 *var* genes with rosetting (Bull, Berriman et al. 2005; Kaestli, Cockburn et al. 2006; Kyriacou, Stone et al. 2006; Warimwe, Keane et al. 2009).

A



B



C.

Clone 17C		Clone 13C	
Sequence	**Number and proportion**	**Sequence**	**Number and proportion**
SA075_1	6 (33 %)	SA075_6	4 (25 %)
SA075_2	6 (33 %)	SA075_7	2 (12.5 %)
SA075_3	2 (11 %)	SA075_8	2 (12.5 %)
SA075_4	2 (11 %)	SA075_9	2 (12.5 %)
SA075_5	2 (11 %)	SA075_10	2 (12.5 %)
		SA075_11	2 (12.5 %)
		SA075_12	2 (12.5 %)
TOTAL	18 (100 %)	**TOTAL**	16 (100 %)

Figure 4.3: *Amplification and transcriptional profiling of DBLα tag sequences of R+ (clone 17) and R- (clone 13) from SA075.* ***A)*** *1 % agarose gel showing products from PCR amplification of the DBLα tag region of SA075 R+ and R- clones. The transcripts were devoid of genomic DNA contamination as seen with the RT- controls that were not treated with reverse transcriptase enzyme. A 3D7 DNA was amplified as a positive control for the PCR while the NTC represented the no-template negative control* ***B)*** *Pie charts showing transcript profiles from R+ and R- clones. Each slice of the chart was numbered to represent unique sequences (listed in Table* ***C)*** *and expressed as a proportion of the total sequences for each clone. The colours represent different Cys/PoLV groups as shown in the key.*

It was not clear why a single dominant gene was not transcribed in the R+ clone as would have been expected. It is possible that: - 1) the rosetting frequency of 44.9% observed in clone 17 was not high enough to give a clear dominant gene and that 2), the number of colonies picked was also not sufficient enough for sequence analysis. Clone 17 was therefore re-cloned in order to: -

1. Try and obtain a single dominant gene from a clone with higher % RF.

2. Establish whether the two dominant genes could have been undergoing switching. It could be that there was a preferential switch from one of these genes to the other over time *in vitro*.

4.4.2: Re-cloning of SA075 R+ (clone 17) parasites

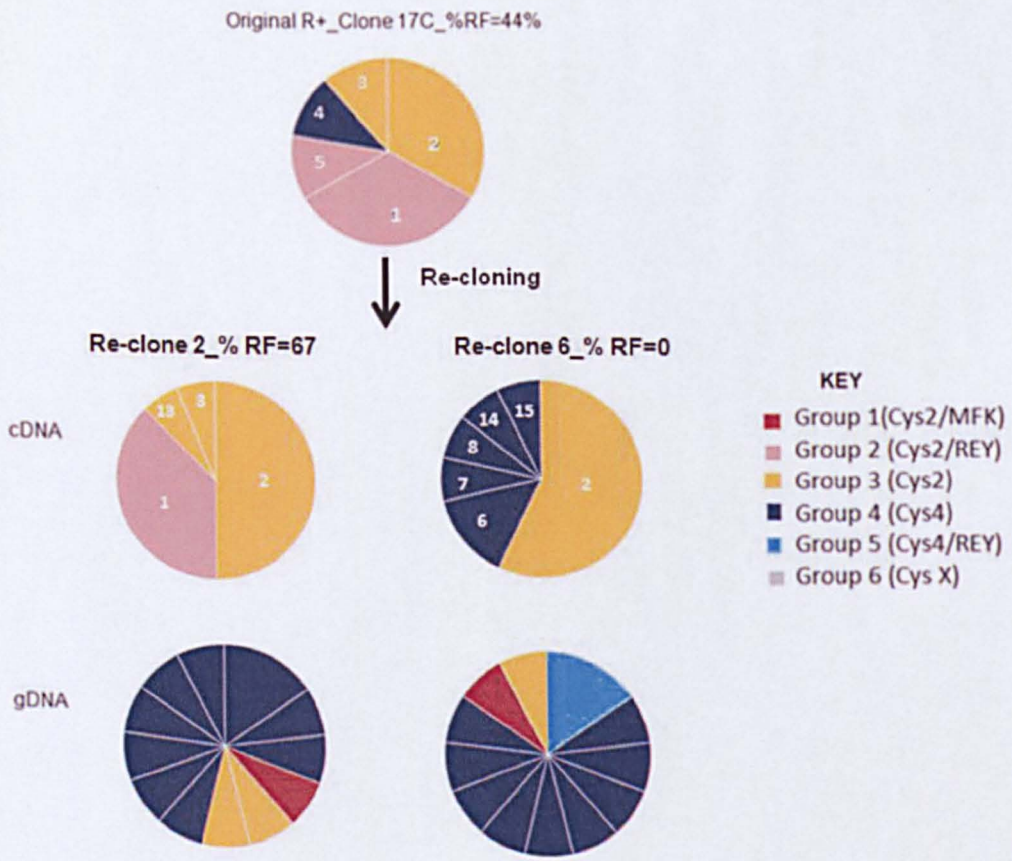
Re-cloning of the clone 17 was done in a similar manner to the first round of cloning as described in the method section of this chapter (section 4.3.2). Of the 60 wells that were inoculated, 10 (17%) that were parasite positive were transferred into culture flasks. At about 5% parasitaemia, the clones were tested for rosetting and agglutination. Hyperimmune serum (A1) was used for the agglutination assay. Out of the 10 clones, 7 were rosetting (range 1.0-52% rosette frequency) and one was agglutinating. The clone with the highest rosetting frequency (Re-clone 2) (52%) and one with 0% (Re-clone 6) were picked for transcript profiling while the remaining clones were frozen down in glycerolyte at -80°C. Note that while the first round of cloning had more clones that were parasite positive with fewer rosetting clones (49%), the re-cloning process had higher proportion (70%) of rosette positive clones.

Table 4.9: *Results from re-cloning of SA075 clone 17 showing the 10 parasite positive clones with their rosetting frequency and agglutination scores. The clones are named "Re-clone" to differentiate them from those of the first round clones.*

Clone ID	%RF	Agglutination
Re-clone 1	32	0
Re-clone 2	52	0
Re-clone 3	1.3	0
Re-clone 4	0	0
Re-clone 5	5.1	0
Re-clone 6	0	0
Re-clone 7	0	0
Re-clone 8	1.8	Agglutinating
Re-clone 9	3.2	0
Re-clone 10	1.0	0

Re-clone 2 (R+) and Re-clone 6 (R-) were picked for RNA isolation and cDNA preparation. Prior to the RNA isolation, the R+ clone was enriched for rosettes by gelatin method up to a rosetting frequency of 67.1 %. A total of 16 transcript sequences were analyzed for R+ with sequences SA075_2 being dominant (8 sequences) while sequence SA075_1 was the second dominant (6 sequences) (Figure 4.4, Panel A and B). However, the isogenic R- clone had SA075_2 hence it was ruled out as the gene responsible for rosetting in SA075 (Figure 4.4, Panel A). Analysis of genomic DNA profiles showed no evidence of primer bias. It was therefore concluded that SA075_1 was the most likely candidate gene involved in rosetting in SA075, and this sequence was renamed SA075*var*1.

A



B

Re-clone_2		Re-clone_6	
Sequence	**Number and proportion**	**Sequence**	**Number and proportion**
SA075_1	6 (37.5 %)	SA075_2	8 (57 %)
SA075_2	8 (50 %)	SA075_6	2 (14 %)
SA075_3	1 (6 %)	SA075_7	1 (7 %)
SA075_13	1 (6 %)	SA075_8	1 (7 %)
		SA075_14	1 (7 %)
		SA075_15	1 (7 %)
TOTAL	16 (100 %)	**TOTAL**	14 (100 %)

Figure 4.4: A) *Pie charts representing the transcript and genomic DNA sequence profiles for Re-clone 2 (R+) and Re-clone 6 (R-), following re-cloning from the original clone17C. The pie charts are colour-coded to represent the different sequence groupings based on cys/PoLV classification (Bull, Berriman et al. 2005). Each slice was numbered to represent unique sequence types (listed in Table* ***B****) as a proportion of the total sequences for each clone. Also shown are genomic DNA sequence profiles that were used to check for primer bias. None of the dominant transcript sequences was dominant in the genomic DNA profiles hence ruling out possibility of primer bias.*

4.4.3: Cloning and sequencing of the full-length SA075var1 gene

Identification of the full-length SA075*var*1gene was done by PCR walking, cloning and sequencing, a strategy that had previously been used to characterize rosetting variants such as *Muz12var1* from the Muz12R+ parasite line (Ghumra, Semblat et al. 2012). The UpsB forward primer was first paired up with a SA075*var*1-specific reverse primer, to produce an ~1.5kb product (Figure 4.6, Panel A), which was cloned and sequenced. UpsA and UpsC primers did not produce any PCR product. Sequencing of the 1.5kb product showed 100% nucleotide sequence

identity in the region that overlapped with the SA075*var*1 tag region suggesting that it was the correct sequence. A comparison of other randomly selected UpsA, B and C *var* gene sequences from Genebank with that of SA075 by sequence alignment (Figure 4.5) showed SA075*var*1 aligning well with the UpsB sequences. The sequences upstream of the initiation codon in SA075*var*1 also had several $polyT_{15-20}$ stretches typical of Ups B sequences (Lavstsen, Salanti et al. 2003) (Nucleotide position 125-145 shown in Figure 4.5).

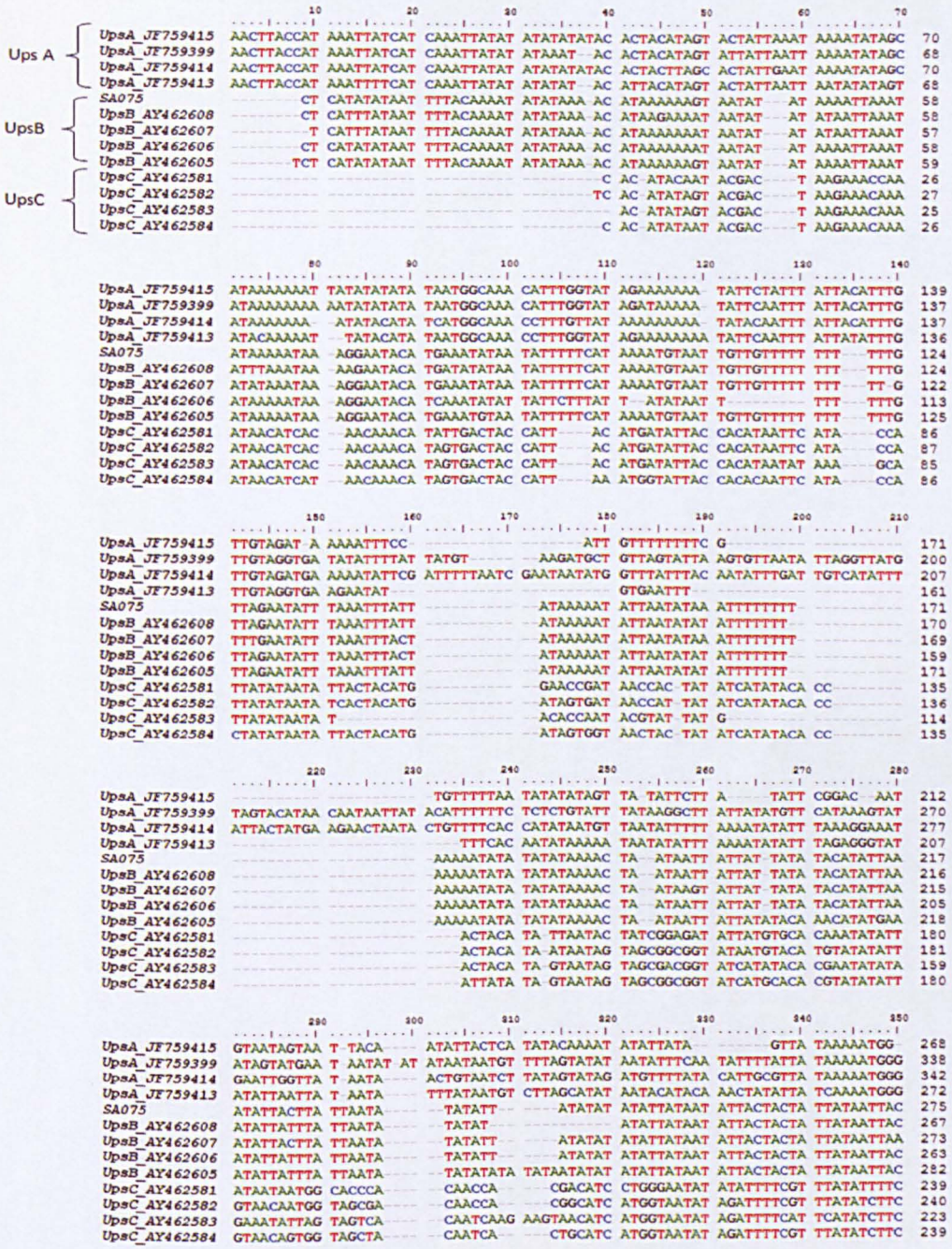


***Figure 4.5**: Partial nucleotide alignment showing approximately 230 bp of 5'UTR region upstream of the initiation codon for SA075var1 against other randomly selected Ups A, B and C var genes. The sequences which were obtained from NCBI database are identified by their accession numbers. SA075var1 aligned well with the Ups B sequences confirming that the var gene is an Ups B type.*

The second sets of PCR were done using SA075*var*1 variant specific forward primer and degenerate reverse primers for DBLβ, γ, δ and ExonII in four separate reactions. The PCR involving the DBLγ primer gave a product of about 6kb (Figure 4.6 B) which was also cloned and sequenced. However due to its large size, only 800bp of the product could be sequenced from both ends. Several sets of internal primers (shown in Table 4.2) were therefore designed from the already sequenced region to further sequence the *SA075var1* gene.

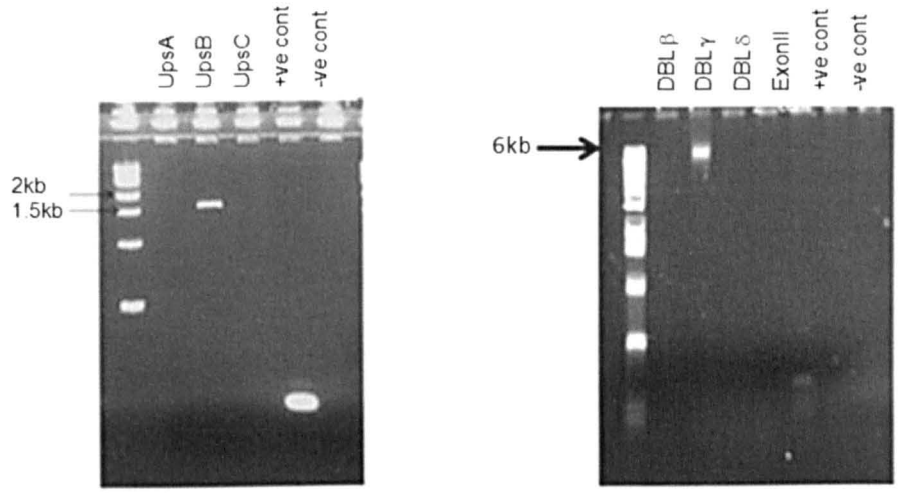
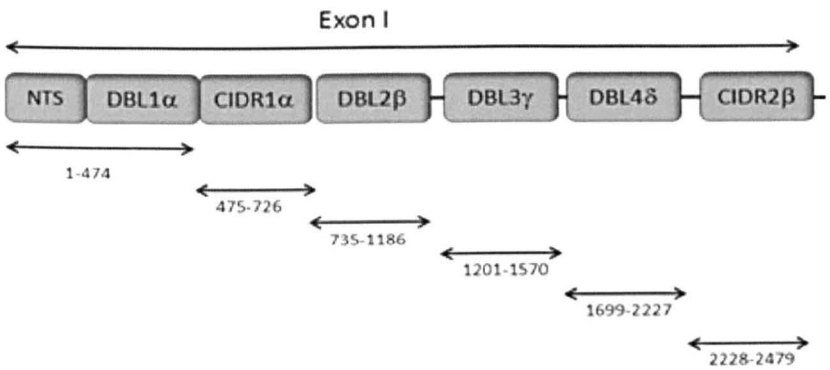


Figure 4.6: A) *2 % agarose gel showing products from PCR involving upstream primers A, B and C with tag specific reverse primers. The Ups B PCR gave an ~1.5kb product which was later cloned and sequenced.* ***B)*** *PCR products from amplification of SA075 DNA using tag specific forward primers paired up with four degenerate primers for DBLβ, γ, δ and exon II in separate PCR reactions. The DBLγ PCR gave a product of ~6 kb which was later cloned and sequenced. However, due to its large size, several rounds of PCRs and sequencing were done both upstream and downstream of the gamma region using designed primers shown in Table 4.2. The molecular weight markers used in gels A and B were a 1Kb ladder from New England Biolabs, UK and nvitrogen™ respectively. An*

amplification involving primers within the DBLα tag region (151bp) was included as a positive control for each PCR.

The full-length gene for SA075*var*1 flanked by the Ups B and Exon II primers (9,613 bp) was finally sequenced (Sequence in Appendix 8.5). The coding region of Exon I (Figure 4.7) starting from the methionine (ATG) to the end of CIDRβ was 7,455 bp long (2485 amino acids). Figure 4.8 shows the final domain architecture of SA075*var*1 Exon I with the domain boundaries derived from the *Var*Dom 1.0 Server (Rask, Hansen et al. 2010).



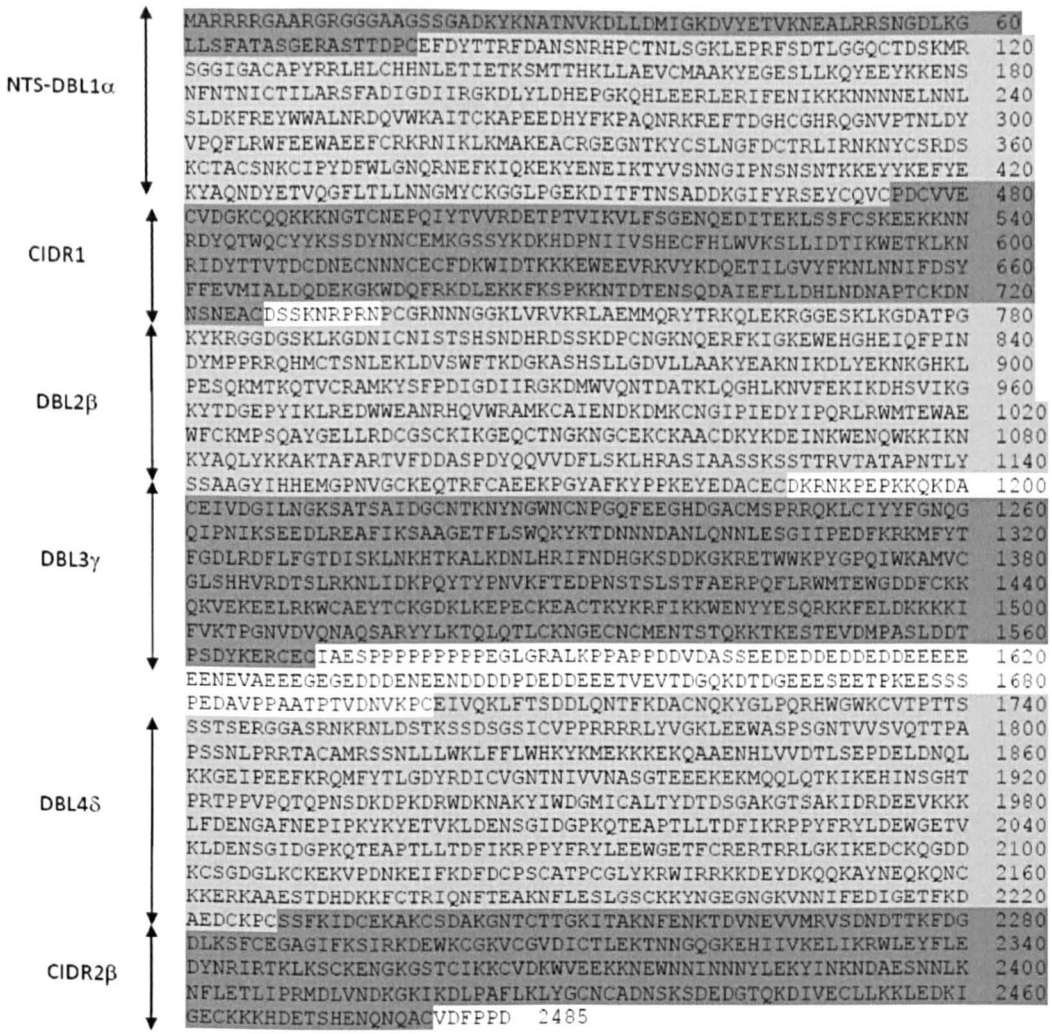


Figure 4.7: Top: *A schematic diagram of the domain architecture of SA075var1 Exon I. The amino acid sequence boundaries for each domain were retrieved from the VarDom 1.0 Server (Rask, Hansen et al. 2010).* ***Bottom.*** *Predicted amino acid sequence for the coding region of SA075var1 exon I.*

4.4.4: Comparison of SA075var1 sequences with other rosetting variants

The SA075*var*1 gene was compared with sequences of other rosette-mediating genes that have already been characterized. These included the proven rosette-mediating variants encoded by IT*var*60, TM284*var*1, HB3*var*6, *ITvar9*, Muz12*var1*,

and *Var O* and the possible rosette-mediating variant *TM180var1*. The comparison was based on overall domain architecture and on sequence features within the DBLα region, which is thought to play a key role in rosetting. Comparison of the DBLα region, was based on the CP classification method as well as block sharing groups (BS) (Bull, Buckee et al. 2008) (discussed in Chapter 1 section 1.2.3.1). A DBLα tag sequence would fall into either BS 1 or BS 2 with BS1 being exclusively group A-like sequences while BS2 contains some non-group A sequences (Bull, Buckee et al. 2008). The parasites were also compared based on their IgM binding phenotype (Ghumra, Semblat et al. 2012), which has previously been associated with rosetting and severe disease in field isolates (Rowe, Shafi et al. 2002). Results of these comparisons are summarized in Figure 4.8 and Table 4.10. Based on the overall domain architecture, *SA075var1* gene was found to be most similar to *TM180var1*, an UpsB gene that was associated with rosetting in transcriptional profiling studies of parasite TM180R+ (described in section 4.1.2). However, a rosetting function for the TM180*var*1 variant has not been confirmed, because antibodies to TM180*var*1 NTS-DBLα did not inhibit rosetting in TM180R+ parasites (Ghumra, Semblat et al. 2012). SA075*var*1 and TM180*var*1 shared not only similar domain architectures but both are also derived from non-IgM binding parasites (Figure 4.8 from Ghumra *et al.,* 2012) (Ghumra, Semblat et al. 2012). Although they belonged to the same BS group 2, they fell into different CP groups 3 and 2 respectively. Pair-wise amino acid identities for the individual domains showed DBL2β to have the highest identity score while DBL4δ had the lowest. The identity scores for the domains were as follows:- NTS-DBL1α-45.88%, CIDR1α-45.04%, DBL2β-57.49%, DBL3γ–36.71%, DBL4δ–20.27%, CIDRβ-45.06%. The overall pair-wise amino acid sequence identity between SA075*var*1 and TM180*var*1 was 43.3%.

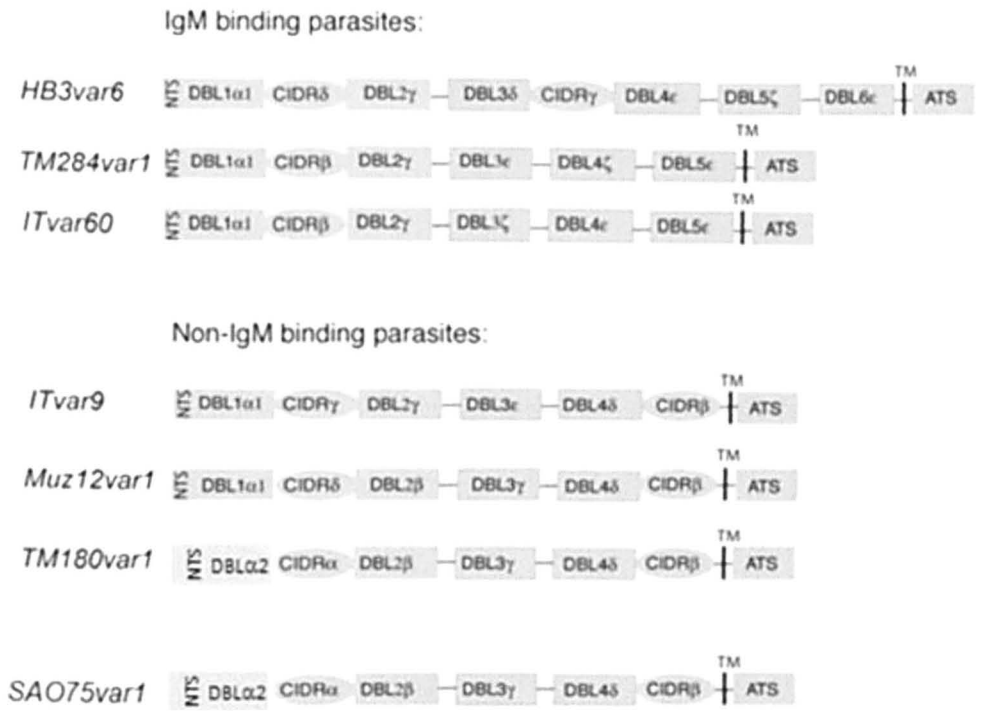


Figure 4.8: *A comparison of domain architectures for different rosetting variants from IgM-binding and non-IgM binding rosetting parasite strains. SA075var1 and TM180var1 have similar domain architectures. Figure from Ghumra et al., 2012 (Ghumra, Semblat et al. 2012).*

Table 4.10*: A comparison of various DBLα-tag sequence features in SA075var1 versus eight rosette mediating variants. The table shows sequence classification features based on methods described by Bull and colleagues(Bull, Berriman et al. 2005; Bull, Buckee et al. 2008).The distinct sequence motifs contain the 4 amino acid motifs at the positions of limited variability (PoLVs), the number of cysteine residues between PoLV 3 and 4 and length of the tag based on amino acid count.*

	Isolate Name	*Pf*EMP1 variant	CP group	Distinct Sequence Motifs (length of tag)	BS_group
1	TM284R+	TM284*var*1	3	MFLP-LRED-NALT-2-PTNL-116	0
2	HB3R+	HB3*var*6	3	LYSG-LRED-RAIT-2-PTNL-126	1
3	IT/PAR+	IT*var*60	6	MFKP-LRED-KAIT-1-PTNL-117	1
4	IT/R29	IT*var*9	1	MFKP-LRED-KAIT-2-PTNL-117	1
5	TM180R+	TM180*var*1	3	LFIG-LRED-DALT-2-LTNL-122	2
6	Muz12R+	Muz12*var*1	3	LYLG-VREA-KALT-2-PTNL-113	1
7	SA075	SA075*var*1	2	LYLD-FREY-KAIT-2-PTNL-111	2
8	3D7	PF13_0003	1	MFKS-LREA-KAIT-2-PTYL-113	1
9	Palo Alto	*Var*O	3	MFLP-LRED-KALT-2-PTYL-117	1

4.4.5: Classification of SA075var1 based on Domain cassette method as described by Rask et al., 2010 (Rask, Hansen et al. 2010)

*Pf*EMP1 sequences have recently been classified into domain cassettes, defined as two or more consecutive domains belonging to particular subclass and present in three or more of the 7 *P.falciparum* genomes that were characterized by Rask *et al.,* 2010 (Rask, Hansen et al. 2010). SA075*var*1 did not seem to fall into any of the 24 domain cassette groups that have been described (SA075 subdomains classified by Lavstsen). However, it had domain cassette 8-like features characterized by Ups B, followed sequentially by a DBLα2, CIDRα, DBLβ and finally a DBLγ domain. The specific sub-domain classes were, however, not of a typical DC8 sequence (Figure 4.9). TM180*var*1, which had an overall domain architecture similar to SA075*var*1, also had domain cassette 8-like features but with different subdomain types. Its architecture was however much more similar to a typical DC8 with a mismatch in only the CIDRα subdomain type, unlike SA075*var*1 that had two mismatches for both CIDRα and DBLγ. Both DC8 and DC13 have recently been implicated in pathogenesis of severe disease (Lavstsen, Turner et al. 2012; Bertin, Lavstsen et al. 2013). Whether SA075*var*1 is important for severe disease will be discussed in Chapter 5.

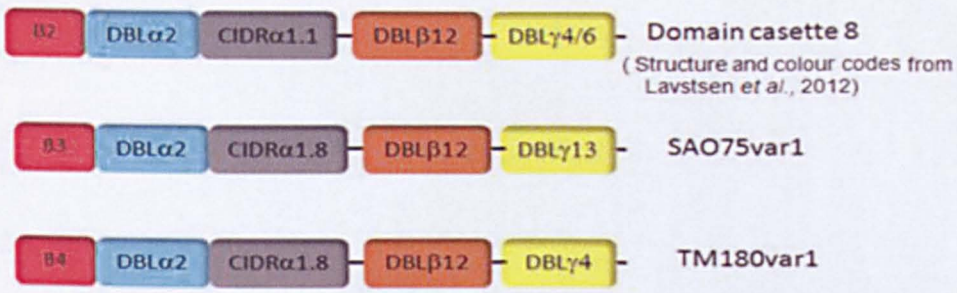


Figure 4.9: *Schematic domain structure for a PfEMP1 containing DC8 as described by Rask et al., 2010 (Rask, Hansen et al. 2010). Based on overall domain architecture, SA075var1 and TM180var1 contain domain cassette 8-like features but with different subdomain classes.*

4.4.6: Results from Protein expression work

4.4.6.1: Cloning of the PfEMP1 constructs

Prior to protein expression, *Pf*EMP1 constructs were cloned into a TA vector followed by a diagnostic digest using restriction enzymes (Table 4.4) to confirm the presence of the insert. An example of such an agarose gel with products for NTS-DBLα construct following the diagnostic digest is shown in Figure 4.10. One of these confirmed products was then cloned into an expression vector for protein expression.

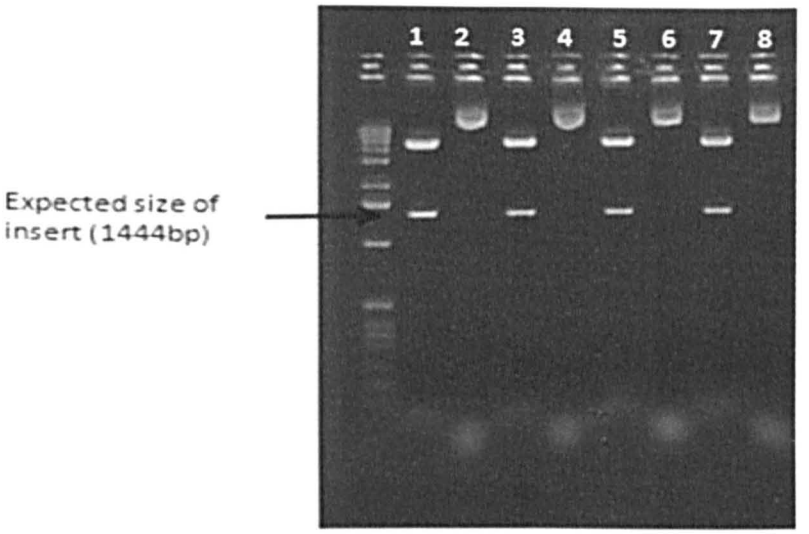


Figure 4.10: *A representative agarose gel stained with ethidium bromide showing products from a diagnostic digest in which plasmids were digested by a restriction enzyme. The 1% gel shows products from a diagnostic digest of plasmids from NTS-DBLα construct. Each paired lane represents the plasmid digested with BamHI/XhoI and an undigested control respectively. The expected product size (1444bp) was present in all the 4 plasmids.*

4.4.6.2: Recombinant proteins from SA075var1 domains

Using the constructs described in Table 4.4, recombinant proteins were made from five single domains and one di-domain of SA075*var*1. CIDR2β did not produce any soluble proteins and was therefore not included in subsequent analysis. The purity of the proteins was checked by Coomassie blue staining described in section 4.3.4.5 under reducing and non-reducing conditions (Figure 4.11). All proteins showed a band at the expected molecular weight although some had degradation or appeared to form aggregations and/or impurities. This however does not affect antibody production or assays based on these antibodies since the responses are variant specific. The NTS-DBLα protein had the highest yield (1.07g/L) and also

required fewer optimizations to obtain soluble proteins. This could possibly be explained by previous data on *Pf*EMP1 protein expression which showed that the inclusion of the NTS to the DBLα domain resulted in expression of soluble proteins (Ghumra, Khunrae et al. 2011). The final protein yields from the 4-8 litre bacterial cultures were follows: 1) NTS-DBL1α2:1.07g/L 2) DBL2β12: 0.66 g/L 3) DBL3γ13: 0.5 g/L 4) DBL4δ:1 g/L 5) NTS-DBL1α2–CIDRα1.8: 0.83 g/L and 6) CIDRα1.8:0.87 g/L.

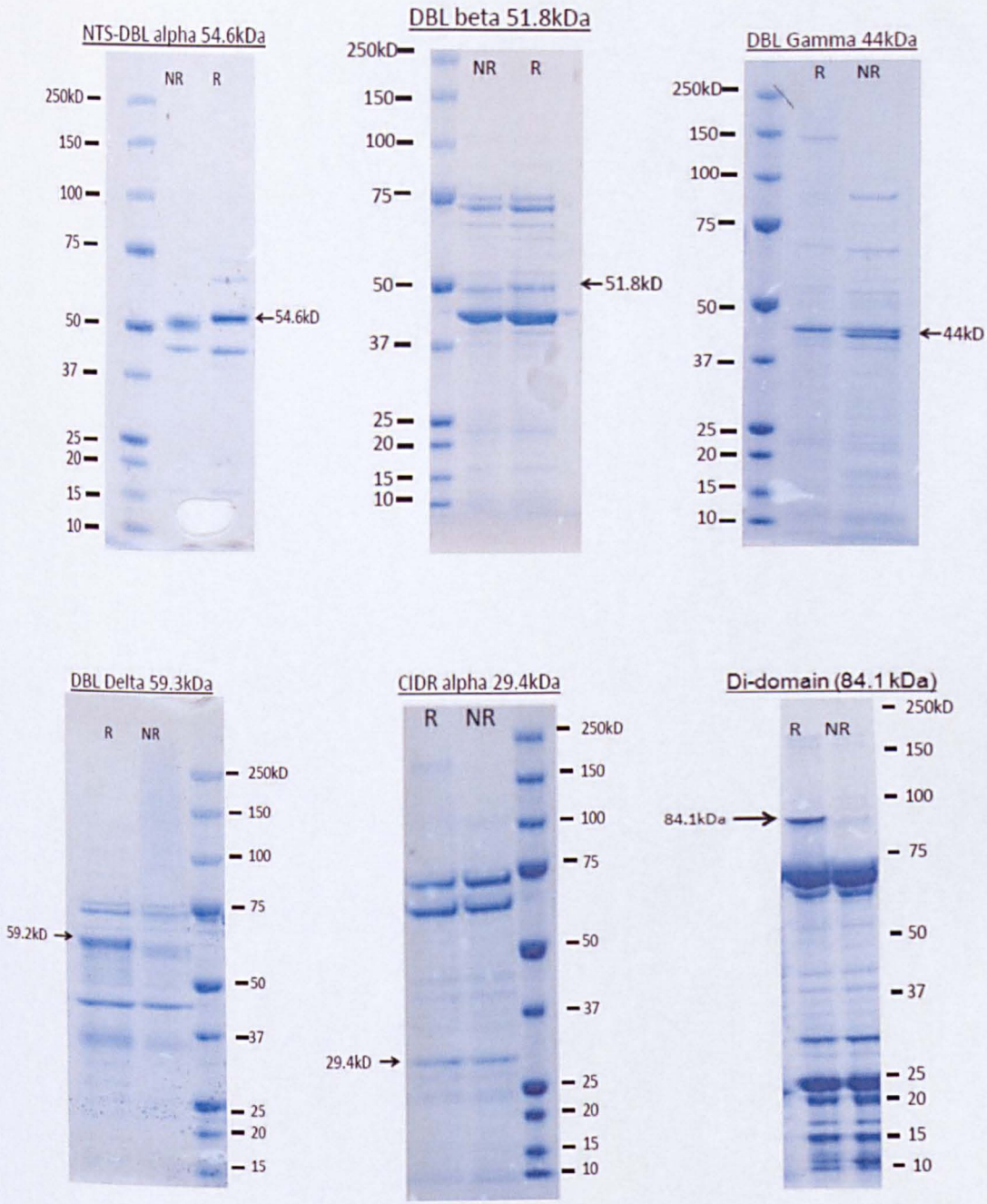


Figure 4.11: *SDS-PAGE showing the predicted molecular masses different recombinant DBL, CIDR and a di-domain from SA075var1 expressed in E.coli. Two micrograms of proteins were run under reducing (R) and non-reducing (NR) conditions on a 4-12% polyacrylamide gel. 5 μl of Precision Plus Prestained Standard (Bio-Rad) was used as the molecular weight ladder.*

4.4.7: Anti-SA075var1 antibodies

Antibody generation was done by BioGenes GmBH (Berlin, Germany). Prior to immunization, pre-immune sera from five rabbits were screened by live cell IFA to check for heterophile antibodies that would react with both infected and non-infected RBCs. The screening assay was done as described in Chapter 2 section 2.3.10. The two rabbits whose pre-immune sera gave the lowest background were selected for immunization with the proteins. Figure 4.12 shows examples of IFA photos for pre-immune screening of sera from two rabbits with different levels of background staining. Serum from rabbit 6 gave punctate fluorescence over both infected and uninfected erythrocytes and was therefore not used for immunization. In contrast, serum from rabbit 7 showed only very pale, smooth background staining similar to that seen with secondary antibody only and therefore rabbit 7 was chosen for immunization.

Immunization was carried out on the two selected rabbits that gave the lowest background using the schedule described in Table 4.7. The anti-sera collected from the 2 rabbits on day 28 was tested by IFA and the antiserum that gave the brightest positive signal was chosen for a final immunization and protein-A purification of total IgG.

Rabbit # 6(with background) Rabbit 7# (without background)

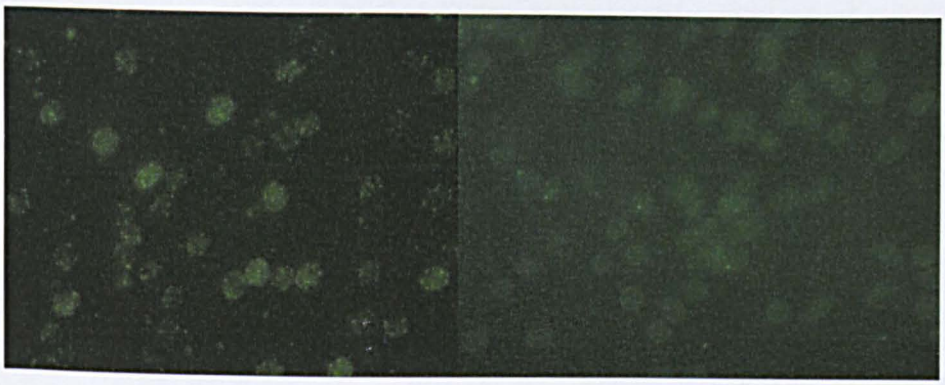


Figure 4.12: *Immunofluorescent assay showing examples of pre-immune sera from two rabbits (6 and 7) tested on RBCs from SA075 culture. Serum from animal 6 gave background and was therefore not used for immunization while serum from animal 7 used due to low levels of background.*

ELISA results from all the six anti-sera at day 28 showed good recognition of the respective antigens with the average antibody titres (defined as titres giving 50 % of the maximum OD) being as follows: NTS-DBL1α 1/30,000; NTS-DBL1α-CIDR1α 1/70,000, CIDR1α 1/20,000; DBL2β 1/30,000; DBL3γ 1/10,000 and DBL4δ 1/35,000. Finally, IgG was purified for all the 6 domains and the final concentration shown in Table 4.11.

Table 4.11: *Final concentration and volume of purified IgG from all the 5 domains and di-domain of SA075var1. Also shown are the ID numbers for the two different rabbits selected for immunization and the ID number for the rabbit from whose serum the IgGs were purified.*

	Domain	ID number for immunized rabbits	Rabbit selected for IgG production	IgG concentration (mg/ml)	Appr. Volume of IgG available in (ml).
1	NTS-DBLα	8062	8063	14.52	11.5
		8063			
2	DBLβ	20876	20877	12.60	23.5
		20877			
3	DBLγ	20137	20137	12.96	17.0
		20138			
4	DBLδ	20488	20489	12.00	18.5
		20489			
5	CIDRα	20878	20879	15.05	20.0
		20879			
6	NTS-DBL1α-CIDR1α (Didomain)	20486	20487	13.20	16.5
		20487			

4.5: DISCUSSION

Identification of *Pf*EMP1 variants that are associated with adhesion phenotypes remains key in understanding the molecular mechanisms of *P.falciparum* sequestration. *Pf*EMP1 variants associated with rosetting have previously been identified and their functional and immunological studies have been extensively carried out (Rowe, Moulds et al. 1997; Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011). Due to the strong association between rosetting and severe disease in field isolates in Sub-saharan Africa (Carlson, Helmby et al. 1990; Rowe, Obeiro et al. 1995; Newbold, Warn et al. 1997; Kun, Schmidt-Ott et al. 1998; Heddini, Pettersson et al. 2001), I sought to identify, clone and sequence a putative full-length *var gene* transcribed by a recently culture-adapted isolate

called SA075, from Kenya whose previous tag analysis had shown sequence features associated with rosetting.

Following two rounds of cloning by limiting dilution and transcriptional profiling of DBLα tag sequences, dominant *var* genes in R+ and R- parasite clones were identified. However, in the first round of cloning, two dominant sequences came up in the R+ clone. Although co-expression of *var* genes as surface antigens within the same parasite has previously been reported (Joergensen, Bengtsson et al. 2010), the most plausible explanation in this case could be that the highest rosette frequency of 44 %, obtained during the first round of cloning, was not high enough to select a single dominant variant. However on re-cloning (% RF-67%), one of the dominant *var* gene (SA075_1) that was identified during the first round of cloning came up in R+ clone and was missing in the R- clone, whereas the second major R+ *var* gene from round 1 (SA075_2) was found to be highly transcribed in the R- clone in round 2. The SA075_1 sequence was therefore deemed to be the most likely rosette-mediating variant, and was selected for further study. It contained two cysteine residues and belonged to Cys/PoLV group 2. Based on these DBL-1 sequence features, the transcript would be grouped as a Group A-like sequence that has previously been associated with rosetting in both lab and field isolates (Bull, Berriman et al. 2005; Kaestli, Cockburn et al. 2006; Kyriacou, Stone et al. 2006; Warimwe, Keane et al. 2009). SA075_1 described in this chapter is the same as the one previously identified in the uncloned SA075 (Bull, Buckee et al. 2008).

The full-length SA075*var*1 gene was successfully cloned and sequenced starting from the tag region and extending the gene both upstream and downstream using degenerate primers. This strategy was previously used by Kraemer et *al.*, 2003

(Kraemer, Gupta et al. 2003) to sequence *Pf*EMP1 genes and has been used to sequence other rosetting variants like Muz12*var*1 (Ghumra, Semblat et al. 2012). Using this method, the SA075*var*1 was found to encode a 5 domain gene with a coding sequence of 7.4 kb starting from the *var* gene translation initiation codon (ATG) up to the last domain of the coding region (CIDR2β) domain. It contained the 5' upstream sequence that was confirmed to be of the Ups B type (Figure 4.5). This method however took several iterations, with some PCRs being unsuccessful and over 20 primer pairs were used. An alternative and faster method would have been whole genome sequencing of SA075 and assembly of the *var* genes as is being done by the next generation sequencing methods in Sanger. The full-length sequence would then be easily identified by blasting the already known tag sequence against the whole genome sequences. However, during the start of the current work, the assembly of *var* genes from whole genome sequencing had not been well established and therefore the PCR walking strategy described above was used.

Features of the SA075*var*1 sequence were compared to those of previously characterized rosette-mediating variants. Interestingly, a comparison of domain architectures showed that SA075*var*1 had a similar architecture to that of TM180*var*1 variant, expressed by a rosetting parasite from S.E Asia, TM180R+. It is important to note that a rosette-mediating function for TM180*var*1 has not been confirmed, and antibodies to TM180*var*1 do not inhibit TM180R+ rosetting (Ghumra, Semblat et al. 2012). Despite the overall sequence identity score of 43.3%, both variants had DC8-like features and have previously been shown to be non-IgM binders (Ghumra, Semblat et al. 2012). It is not known if both parasites use similar receptors on uninfected cells. The genes encoding DC8 are thought to be prevalent in the parasite population and were present in six of the seven

sequenced genomes that were analyzed by Rask *et al.,* 2010 (Rask, Hansen et al. 2010). They are characterized as having an Ups B promoter and contain 4 domains in tandem. Despite the high level of conservation across parasite genomes, the DC8 domains are not identical but share between 45% and 63% similarity at the amino acid level (Lavstsen, Turner et al. 2012), similar to what was seen for SA075*var*1 and TM180*var*1. Chapter 5 will explore whether the SA075*var*1 and TM180*var*1 encode proteins eliciting cross-reactive antibody responses. Recently, *var* genes with DC8 features have been associated with severe disease (Lavstsen, Turner et al. 2012). Interestingly, all the *var* genes with DC8 features were of the Ups B type. It would also be important to study the clinical relevance of SA075*var*1 despite not having typical DC8 features.

Heterologous expression of recombinant proteins has for long been used in studying the antigenicity and other functional characteristics of proteins. However, expression of *Pf*EMP1 proteins, especially as full-length proteins, is difficult due to the high molecular weight of the protein. Most studies on recombinant expression of *Pf*EMP1 have thus focused on single or di- domains which are compatible with most expression systems. However, biochemical and physical characteristics, such as a high number of disulphide bridges still make recombinant expression difficult (Victor, Bengtsson et al. 2010). One study has however managed to express *Var*2csa as a full-length *Pf*EMP1 protein (Khunrae, Dahlback et al. 2010) in an insect expression system *Trichoplusia ni* following codon optimization.

SA075*var*1 domains were expressed as soluble recombinant proteins in *E.coli* to be used mainly for polyclonal antibody production. I have described the inclusion of a heat shock protocol that was able to enhance expression of soluble proteins for some of the SA075*var*1 domains. This strategy has previously been shown to improve the level of expression of recombinant proteins in a soluble native form is

by increasing the cellular concentration of osmolytes or of chaperones (Oganesyan, Ankoudinova et al. 2007). However, CIDRβ in SA075*var*1 could not be expressed as soluble proteins as it remained insoluble even after bacterial cell lysis. Based on the quality of the gels there was evidence of degradation and/or aggregated material or possible contaminating *E.coli* proteins within the recombinant protein preparations. However, some protein at the expected molecular weight was seen in most cases, which shifted upon reduction showing the presence of disulphide bonds (Figure 4.11). This suggests the likelihood that some correctly-folded protein is present, and it can be hoped that antibodies recognizing native *Pf*EMP1 on the surface of live infected erythrocytes will be obtained.

Antibody production was successfully done for all the recombinant domains of SA075*var*1. Whether these antibodies elicit functional and crossreactive antibodies will be discussed in the next chapter. A similar study was done by Ghumra *et al.,* 2010 (Ghumra, Khunrae et al. 2011) on IT*var*9, a rosetting variant in R29 strains in which the antibodies were found to have rosette inhibiting and phagocytosis- mediating abilities. Also of importance will be to find out the functional abilities of antibodies to other extracellular domains other than NTS-DBLα in SA075*var*1. The antibodies were used for selecting for mono-variant rosetting parasites by cell sorting and the parasites were used to explore the clinical relevance of rosetting parasites, as will be discussed in chapter 6.

In conclusion, SA075*var*1, a candidate gene that may encode the rosetting-mediating ligand in SA075 parasite was cloned and sequenced. Recombinant proteins were made from the different domains and antibodies were raised from

the different domains. As has been done for laboratory isolates associated with rosetting eg R29 (Ghumra, Khunrae et al. 2011), Palo alto (Vigan-Womas, Guillotte et al. 2008) and FCR3S1.2 (Albrecht, Moll et al. 2011), the functional and immunological characterization of antibodies to SA075*var*1 recombinant domains will be done and discussed in Chaper 5.

CHAPTER 5

Functional Characterization of anti-SA075*var*1 antibodies

5.1: INTRODUCTION

5.1.1: Anti-PfEMP1 antibodies

Plasmodium falciparum erythrocyte membrane protein 1 *(Pf*EMP1) is an important immune target and would potentially be a good candidate for vaccine development (reviewed Beeson *et al.*,2013 (Beeson, Chan et al. 2013)). An effective anti-*Pf*EMP1 vaccine would be one that ultimately leads to immune clearance of infected RBCs and hence protects against the disease and its consequences rather than against infection *per se*. Also of importance would be a vaccine that induces not only variant specific (Vigan-Womas, Guillotte et al. 2011) but also cross reactive responses, as has recently been reported for anti-rosetting antibodies (Ghumra, Semblat et al. 2012) and anti-CSA binding antibodies in placental associated malaria (Avril, Cartwright et al. 2010; Bigey, Gnidehou et al. 2011).

Many functional and immunological studies have therefore been carried out on antibodies against *Pf*EMP1 variants that are linked to virulent phenotypes. Such studies have focused on single, di-domains or even full-length *Pf*EMP1 to explore the ability of the proteins or their antibodies to inhibit or reverse adhesion. *Var*2CSA which mediates sequestration of parasites in the placenta is among the most characterized *Pf*EMP1 variants. It is the first *Pf*EMP1 in which the full-length gene has been expressed as a recombinant protein (Khunrae, Dahlback et al. 2010) and the crystal structure for DBL3X (Higgins 2008) and DBL6ε domains have been solved. Antibodies against different domains of *var*2CSA have shown varied inhibitory activities in both a strain specific and strain transcending manner

(Fernandez, Kviebig et al. 2008; Nielsen, Pinto et al. 2009; Avril, Cartwright et al. 2010; Gangnard, Tuikue Ndam et al. 2010; Khunrae, Dahlback et al. 2010; Salanti, Resende et al. 2010; Srivastava, Gangnard et al. 2010; Avril, Cartwright et al. 2011). Other adhesion phenotypes whose *Pf*EMP1 variants have been studied include binding to CD36 (Baruch, Gormely et al. 1996), Intracellular adhesion molecule 1 (ICAM-1) (Smith, Craig et al. 2000), platelet endothelial adhesion cell molecule 1 (PECAM1) (Chen, Heddini et al. 2000) and rosetting (Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011; Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012). This chapter focuses on characterization of antibodies to the SA075*var*1 *Pf*EMP1 variant described in Chapter 4.

5.1.2 Functional characterization of anti-PfEMP1 antibodies against rosetting parasites

Like in *var*2csa, the possibility of a limited subset of rosette-mediating variants raises the prospects of an anti-rosette vaccine that would inhibit or disrupt rosette formation. There is sufficient evidence from field studies showing the presence of anti-rosetting activity in serum from malaria patients (Carlson, Helmby et al. 1990; Treutiger, Hedlund et al. 1992; Barragan, Kremsner et al. 1998). Coupled with the various laboratory-based studies on characterization of anti-*Pf*EMP1 antibody responses against rosette-mediating parasites (Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012), it is evident that there are functional antibodies against rosette-mediating variants. Three of the well characterized rosetting variants i.e. Var O(Vigan-Womas, Guillotte et al. 2008), FCR3S1.2*var*2 (ITvar60) (Albrecht, Moll et al. 2011) and IT*var*9 (Ghumra, Khunrae et al. 2011) have all shown rosette-disrupting ability of their anti-DBL1 antibodies against rosettes in homologous parasites. Antibodies to rosetting variants have also been used to explore the antigenic relationship of rosetting parasites. Two studies by Ghumra *et*

al., 2011(Ghumra, Khunrae et al. 2011) on the R29 clone and by Vigan-Womas *et al.,* 2011(Vigan-Womas, Guillotte et al. 2011) on Palo Alto, R29 and 3D7 all showed variant-specific surface–reactive antibodies. However, a follow-up study by Ghumra *et al.,* 2012 (Ghumra, Semblat et al. 2012) showed induction of strain transcending surface reactive antibodies that were not only able to inhibit rosette formation but also meditated phagocytosis in heterologous parasites. However, the cross reactivity seemed to be limited to IgM-binding rosetting parasites, a phenotype thought to be virulence enhancing either by masking *Pf*EMP1 from recognition by specific antibodies or aiding in the stabilization of rosettes (Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011; Ghumra, Khunrae et al. 2011).

While the DBLα domain of the *Pf*EMP1 molecule is thought to be important in mediating rosetting (Rowe, Moulds et al. 1997; Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011), little is known about the role of other domains of the rosette-mediating *Pf*EMP1 variants. Very few studies have been done to characterize antibodies raised against other extracellular domains of rosette mediating variants. Recently, Ghumra and colleagues (Ghumra, Khunrae et al. 2011) studied domain-specific antibody responses, targeted against domains downstream of NTS-DBL1α in IT*var*9 of R29 strain. Results showed that antibodies to all *Pf*EMP1 domains of IT*var*9 recognized the surface of live infected RBCs. Antibodies to all domains except the second DBL domain (DBL2γ) inhibited rosettes and mediated phagocytosis in a strain specific manner. These findings cannot however be generalized to all rosetting variants and it is imperative to study downstream domains of other rosetting variants to understand the pattern of recognition and functionality.

This chapter discusses results on functional characterization of antibodies to SA075*var*1. The *Pf*EMP1 variant was identified as a putative gene involved in rosetting in a recently culture-adapted field isolate called SA075 (Chapter 4). Subsequent experiments however failed to confirm the role of SA075*var*1 in rosetting (see later in this chapter). However, other features of this sequence made it suitable for further continued study. Using the recent *Pf*EMP1 classification method of domain cassettes (DCs) (Rask, Hansen et al. 2010), SA075*var*1 had DC8-like features (Chapter 4, section 4.4.5). With DC8 and DC13 being linked to severe malaria in studies done inTanzanian and Benin (Lavstsen, Turner et al. 2012; Bertin, Lavstsen et al. 2013), the functional characterization of SA075*var*1 was of interest. Polyclonal antibodies were raised in rabbits that were immunized with recombinant protein from five single domains and one di-domain of SA075*var*1. Details on identification of the full-length gene, making of recombinant proteins from the different domains and raising of the antibodies have been described in Chapter 4.

5.2 CHAPTER AIMS

The aim of this chapter was to examine the functional and immunological characteristics of antibodies to the extracellular domains of SA075*var1*. Rabbit polyclonal antibodies were raised against five single domains and one di-domain recombinant proteins of SA075*var*1 as described in Chapter 4 (section 4.4.6). The NTS-DBL1α antibody was further used in developing a cell sorting protocol that was used to select for monovariant-enriched cultures in SA075. This protocol was later adapted for use on other *P.falciparum* strains. Five questions that formed the specific objectives of this chapter were: -

1. Do antibodies raised against extra-cellular domains of SA075*var*1 recognize the surface of homologous parasites?
2. What is the sensitivity/end titre of recognition by these antibodies?

3. Do the antibodies have the ability to induce phagocytosis?
4. Do these antibodies cross react with other *P.falciparum* laboratory strains?
5. Is there a correlation between the parasites rosetting frequency and the percent positive population when stained by homologous antibodies?

5.3 MATERIALS AND METHODS

The methods used in this section were previously described in Chapter 2. These include: -1) antibody staining by FACS and IFA assays 2) antibody-mediated phagocytosis assay. Other assays used this chapter include:- Trypsinization experiments and cell sorting using homologous NTS-DBLα antibodies.

5.3.1: Trypsinization experiments

*Pf*EMP1 has previously been shown to be sensitive to trypsin treatment (Gardner, Pinches et al. 1996). To check whether the anti-SA075*var*1 antibodies were targeting the *Pf*EMP1 molecule, infected RBC were treated with trypsin prior to antibody staining. 20 µl of trophozoite stage parasites at greater than 5% parasitaemia were incubated in 500 µl of 10µg/ml of TPCK-trypsin (Sigma) for 5 minutes at room temperature. A mock trypsin treatment in incomplete RPMI was also included. The reaction was then stopped by addition of 500 µl of 1 mg/ml of soybean trypsin inhibitor (Sigma) to both the trypsin-treated and mock trypsin-treated samples followed by 5 minute incubation at room temperature. The cells were then washed and stained for FACs assays as described in Chapter 2.Section 2.3.10.

5.3.2: Use of NTS-DBL α antibodies to select for monovariant parasite population by cell sorting.

Heterogeneity of parasite populations due to antigenic switching is a common feature of *in vitro* culture in *P.falciparum*. In rosetting parasites, it results in different rosette-forming parasites expressing different *Pf*EMP1 variants in a single culture (Vigan-Womas, Guillotte et al. 2008; Vigan-Womas, Guillotte et al. 2011) which may confound studies on genotype-phenotype associations. A cell sorting protocol done by FACS was optimized to select for a homogenous parasite population expressing a single variant. This protocol was developed with SA075 and then adopted and used to sort *P. falciparum* strains including PAR+, TM284R+, HB3R+, R29+, TM180R+ and Muz12R+.

The cell sorting protocol involved 3 major steps: -

i. Enrichment of trophozoite stage parasites to greater than 80% parasitaemia by magnetic-activated cell separation (MACS) prior to antibody staining. This substantially reduces the content of uninfected RBCs and cell debris in the pre-sorting material, and subsequently shortens the length of time taken for sorting.

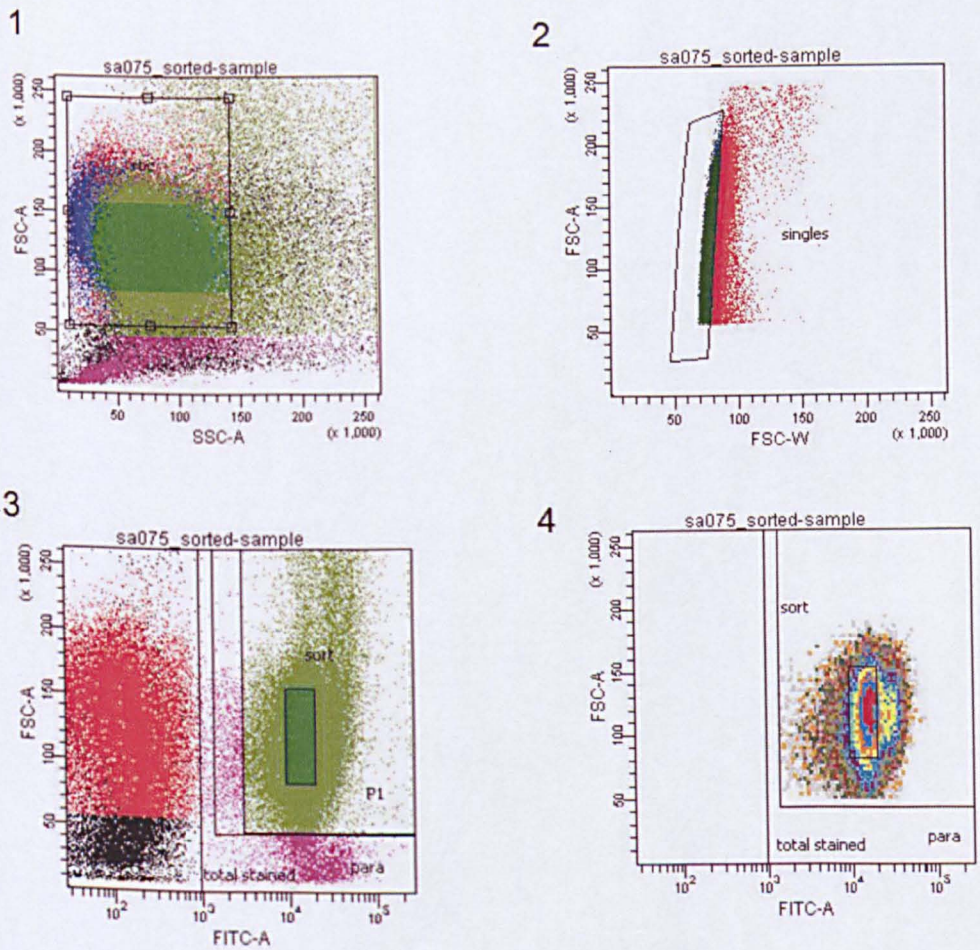
ii. Staining of parasites with homologous NTS-DBLα antibodies.

iii. Cell sorting on a FACSAria (BD Biosciences) and re-culturing of the sorted cells.

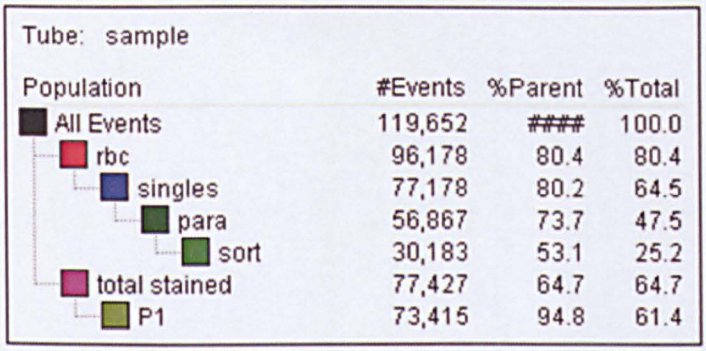
Enrichment of parasites was first done by spinning down the culture suspension and washing it twice in incomplete RPMI. Following the washes, the culture was resuspended back to its original volume using incomplete RPMI supplemented with 200μg/ml of Fucoidan (Sigma) to keep any rosettes disrupted. The culture

suspension was then carefully passed though a magnetic column that had been pre-equilibrated with incomplete RPMI with Fucoidan and the flow-through dispensed in a dropwise manner. This allowed for maximum separation of the hemozoin-rich trophozoites by the magnetic column. The flow-through was then passed through the column for a second time to ensure that all the trophozoites were captured. The column was detached from the magnet and MACS purified trophozoites were collected by passing the incomplete RPMI/Fucoidan media through the detached column. The final pellet volume of between10-20 μl had a parasite count of greater than 80%. These were then washed three times in 1X PBS, after which they were resuspended in 3ml of 1X PBS/1% BSA with 25μg/ml of Fucoidan in a 15ml Falcon tube. A smaller volume of 500μl suspension was kept in a separate 15ml tube to be used as a negative control during gating. Homologous NTS-DBL1α antibody was added to the 3 ml suspension to a final concentration of 25μg/ml. Note that the control tube was not stained with the primary antibody. This was incubated at room temperature for 45 minutes with regular flicking of the tube after every 10 minutes to in order to keep the cells in suspension. The culture was washed three times in 3ml sterile 1XPBS by centrifuging at 2000rpm for 2 minutes. 3 ml of secondary antibody Alexa Fluor 488-labelled goat anti-rabbit IgG (catalogue number A11034, Invitrogen Ltd, Paisley, UK) diluted at 1:1000 in PBS/1% Ig-free BSA/25μg/ml of Fucoidan was then added to both the sample tube and the control tube. This was again incubated for 45 minutes at room temperature while covered in foil to protect it from light. Flicking of the tube was repeated every 10 minutes. Following the incubation, 3 washes were done in sterile 1XPBS and the pellet finally re-suspended in 3 ml culture medium containing 25μg/ml of Fucoidan. Meanwhile a collection tube of 5ml warm culture medium with 50% serum was prepared in which the sorted parasites would be collected. Sorting was done on FACSAria (BD

Biosciences) cell sorter. Prior to sorting, an RBC gate was set on a forward scatter (FSC) and side scatter (SSC) (Figure 5.1, panel 1). The gated region represented a tight population of cells in which dead cells and debris were excluded. Next, the forward scatter-width (FCS –W) parameter was used to gate out the double or multiple cells from single cells to avoid the possibility of stained cells sticking together with unstained cells (Figure 5.1, panel 2). The singlets were then gated based on FITC stain to separate the stained population from the unstained population (Figure 5.1, panel 3) and finally a sorting gate was set within the stained population (Figure 5.1, panel 4). The gating strategy is summarized in Figure 5.1. Cell sorted parasites were then washed and re-cultured as described in Chapter 2, section 2.3.1.



5



Tube: sample

Population	#Events	%Parent	%Total
All Events	119,652	####	100.0
rbc	96,178	80.4	80.4
singles	77,178	80.2	64.5
para	56,867	73.7	47.5
sort	30,183	53.1	25.2
total stained	77,427	64.7	64.7
P1	73,415	94.8	61.4

Figure 5.1: *An overview of the gating strategy used for cell sorting of rosetting parasites using homologous NTS-DBL1α antibodies.* ***1)*** *A tight RBC gate was first set on FSC and SSC scatter in order to exclude debris and/or dead cells* ***2)*** *Next, FCS (W) parameter was used to gate out the double or multiple cells from single cells* ***3)*** *The singlets were then gated based on FITC stain to separate the stained population from the unstained population.* ***4)*** *It is within the stained cells that the sorted population was gated.* ***5)*** *An output from the FACS sorting machine showing a summary of the sorting results including the proportion of each population. The different colors represent different cell populations. All events (100%) were first acquired as represented in black colour. Rbcs (in red) represents the proportion of cells following the first gating to remove debris/dead cells. Single cells (in blue) were then gated before the stained population (deep green) were gated. A subpopulation of the stained cells was then sorted (called 'sort' and represented in green). "Total stained" (purple colour) represents all stained cells including singlets and doublets while P1 is a subset of the stained parasites, but which also includes singlets and doublets.*

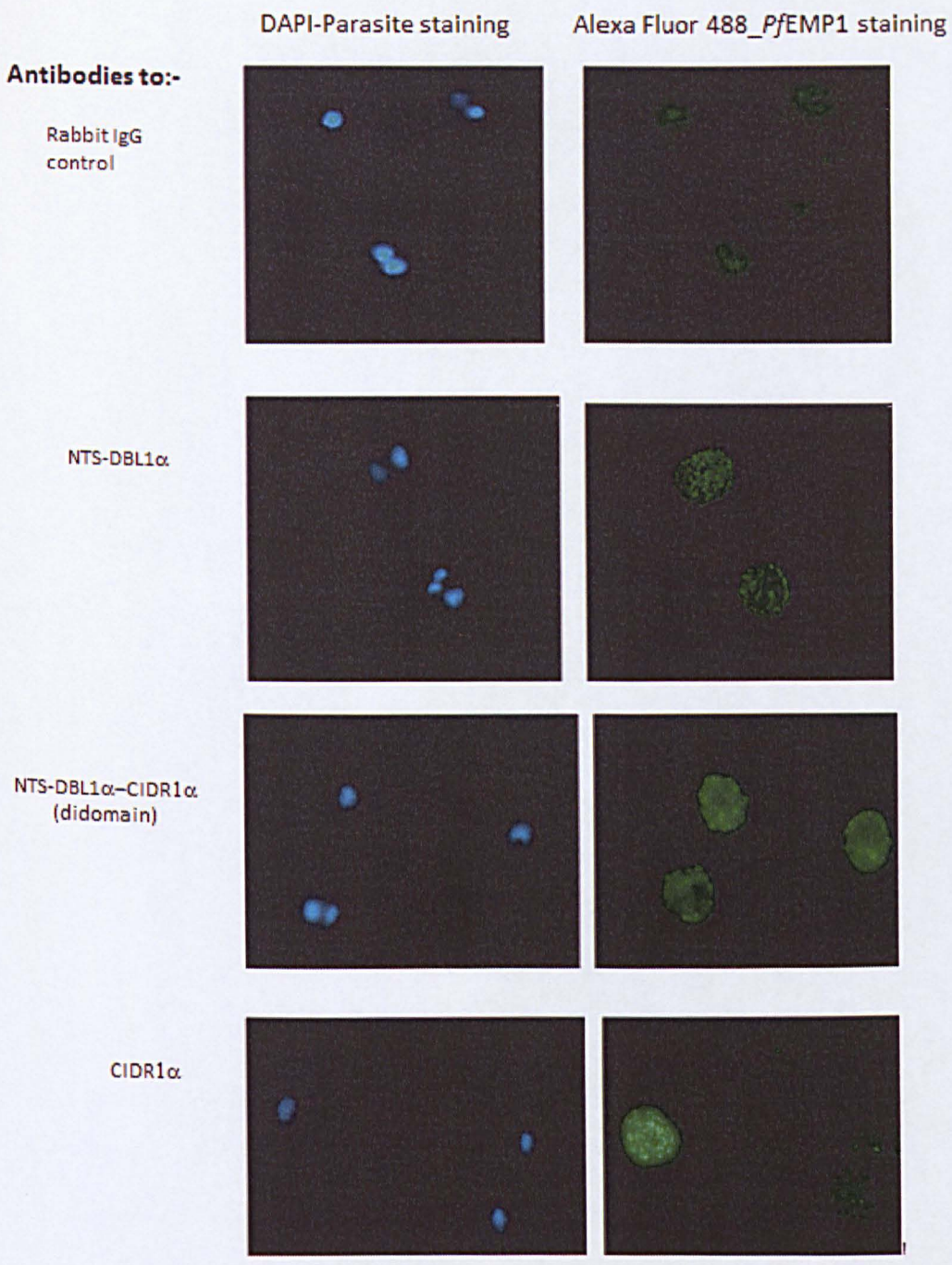
5.4: RESULTS

5.4.1: Antibodies to the five single domains and one di-domain of SA075var1 recognize the surface of SA075 infected RBCs

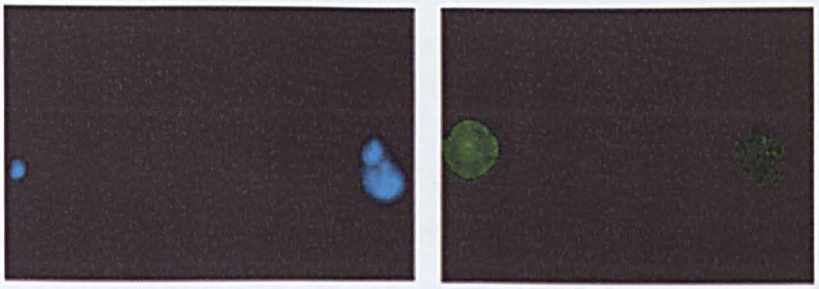
IFA and FACS assays were carried out on live infected RBCs to determine whether the polyclonal rabbit IgGs recognized native *Pf*EMP1 on the surface of SA075 parasites. For all the six anti-*Pf*EMP1 antibodies, IFA slides showed a positive signal which appeared as dotty punctate fluorescence pattern, similar to previously published results on *Pf*EMP1 staining (Vigan-Womas, Guillotte et al. 2008; Albrecht, Moll et al. 2011; Ghumra, Khunrae et al. 2011) (Figure 5.2 A). Attempts to visualize intact rosettes on the stained parasites were however unsuccessful. The negative control i.e.IgG from a non-immunized rabbit, showed a pale background fluorescence indicative of lack of binding. All IFA slides were viewed with a 100X objective using a Leica DM 2000 fluorescent microscope

FACS data also showed similar specific surface reactivity for all the six SA075*var*1 antibodies, which was absent in the control antibody (Figure 5.2B and C). Infected RBCs were stained with Hoechst 33342 (Sigma) while anti-*Pf*EMP1 reactivity was visualized by Alexa Fluor 488 Goat anti-Rabbit IgG. Both the anti-SA075*var*1 antibodies and the rabbit IgG control antibody were used at a concentration of 400μg/ml. Figure 5.2B and C show FACS plots for results for the staining of the samples and the control.

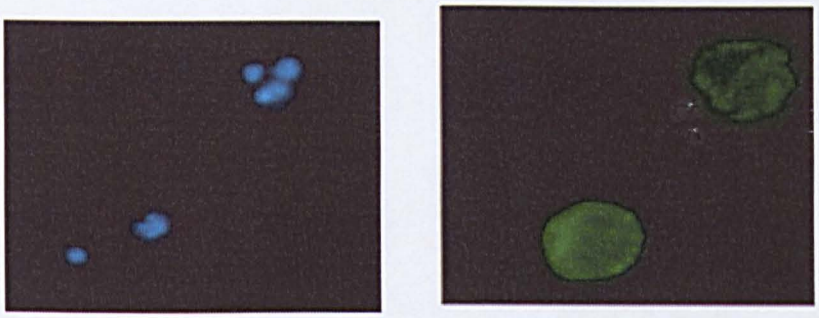
A)


DAPI-Parasite staining
Alexa Fluor 488_PfEMP1 staining
Antibodies to:-
Rabbit IgG control
NTS-DBL1α
NTS-DBL1α–CIDR1α (didomain)
CIDR1α

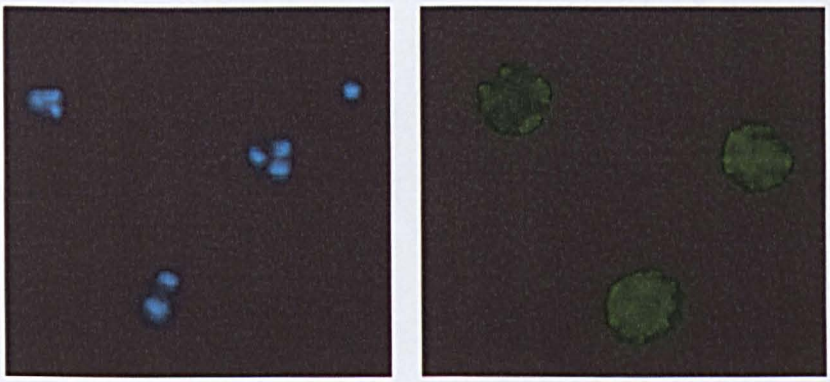
DBL2β



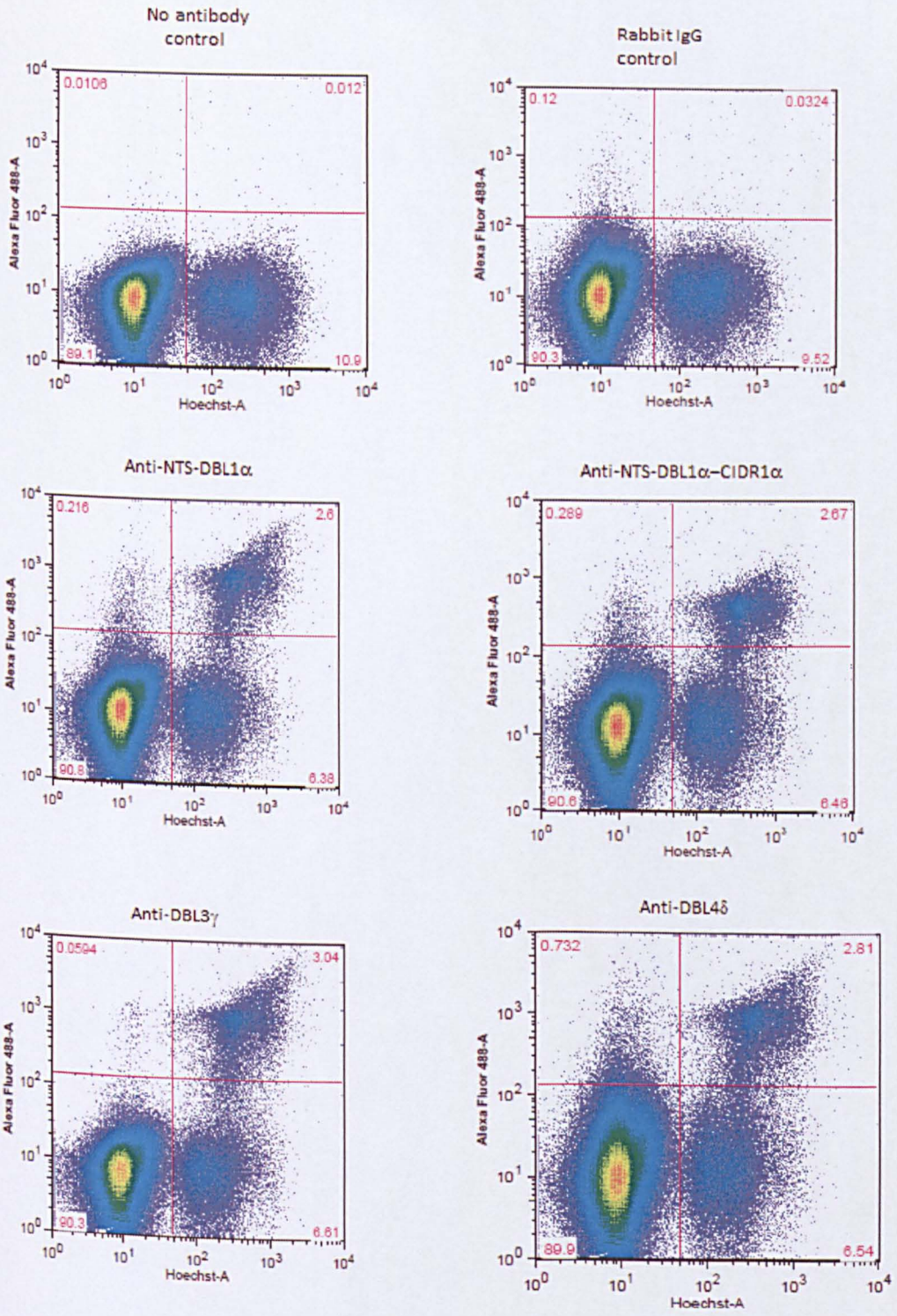
DBL3γ



DBL4δ



B)


No antibody control
0.0106
0.012
89.1
10.9
Rabbit IgG control
0.12
0.0324
90.3
9.52
Anti-NTS-DBL1α
0.216
2.6
90.8
6.38
Anti-NTS-DBL1α–CIDR1α
0.289
2.67
90.6
6.46
Anti-DBL3γ
0.0594
3.04
90.3
6.61
Anti-DBL4δ
0.732
2.81
89.9
6.54
Alexa Fluor 488-A
Hoechst-A

C)

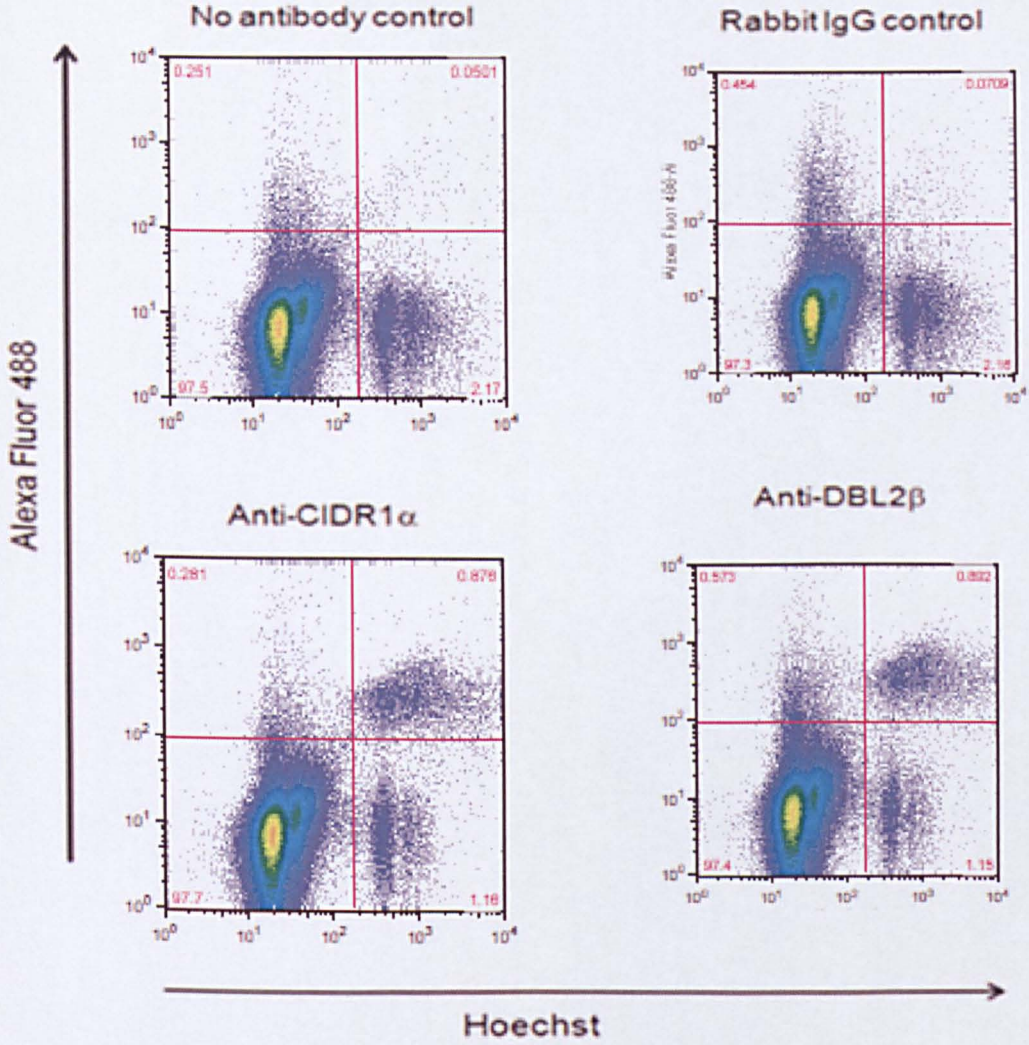


Figure 5.2: A) *IFA photos showing surface staining of live infected RBCs by the six homologous SA075var1 antibodies and a rabbit IgG control antibody. Infected RBCs were stained with DAPI and anti-PfEMP1 reactivity was visualized by Alexa Flour 488 dye diluted at 1:1000. Specific staining appeared as dotty punctate fluorescence pattern while the control showed a pale background fluorescence* ***(B)*** *FACS plots showing the recognition of PfEMP1 on the surface of SA075 infected RBCs by 4 of the 6 homologous SA075var1 antibodies at 400μg/ml. These were antibodies against NTS-DBL1α, NTS-DBL1α–CIDR1α, DBL3γ and DBL4δ.*

Infected RBCs were stained with Hoechst while PfEMP1 was visualized by secondary antibody Alexa Flour 488 diluted 1:1000. Also included in the assay were two negative controls:-one without primary antibody and one in stained with 400μg/ml of rabbit IgG. ***C)*** *2 of the 6 antibodies against CIDR1α and DBL2β analyzed as in* ***B*** *above but on a different day.*

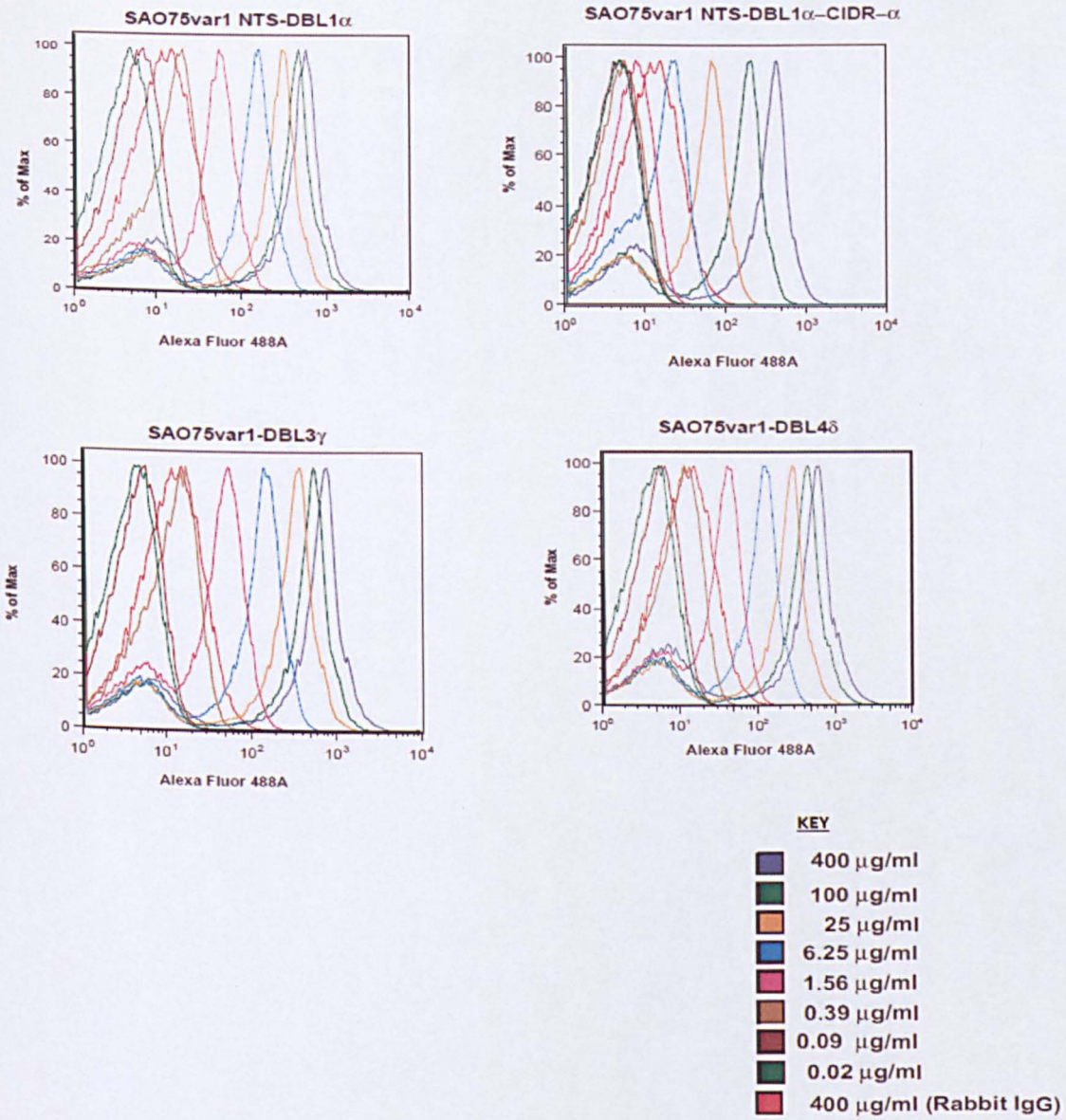
5.4.2. Limit of dilution/sensitivity of anti-SA075var1 antibodies

The limit of dilution or end titre for all the six anti SA075*var*1 antibodies was determined by flow cytometry. This is different from a previous study (Ghumra, Khunrae et al. 2011) where the end titres of anti-*Pf*EMP1 antibodies to R29 parasites was determined by IFA as the lowest concentration at which >50% of the infected RBCs in the culture showed punctate fluorescence. In this study, SA075 parasites were incubated with a four-fold dilution of purified IgG starting with 400μg/ml then 100, 25, 6.25, 1.57, 0.39, 0.10 and 0.03 μg/ml and analysed by FACS. The end titre was defined as the lowest concentration giving a positive signal above the rabbit IgG control as shown in the histograms (Figure 5.3, panel A and B).

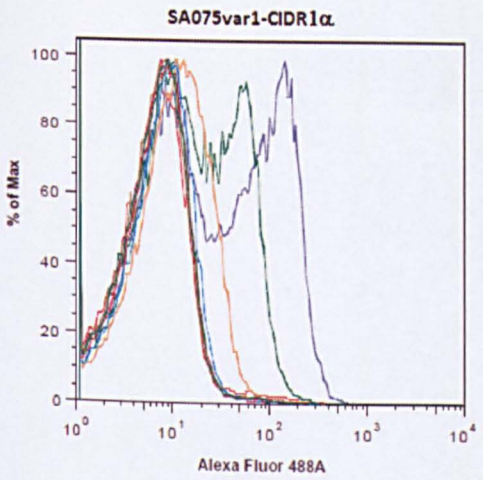
Results showed end titre for the anti-SA075*var*1 antibodies as follows: - anti-NTS-DBL1α (1.57 μg/ml), anti-NTS-DBL1α-CIDR1α (25 μg/ml), anti-CIDR1α (400 μg/ml), anti-DBL2β (25 μg/ml), anti-DBL3γ (1.57 μg/ml) and anti-DBL4δ (6.25 μg/ml) (Figure 5.3 panel A and B). A different comparative analysis method to determine the end titre concentrations for the different antibodies was done using a FlowJo software (Tree star Inc.) with both methods showing similar results. In this latter analysis, the percentage positive population was calculated using a tool called the Overton cumulative histogram subtraction method, where the proportion of the negative control population i.e. rabbit IgG was subtracted from each sample

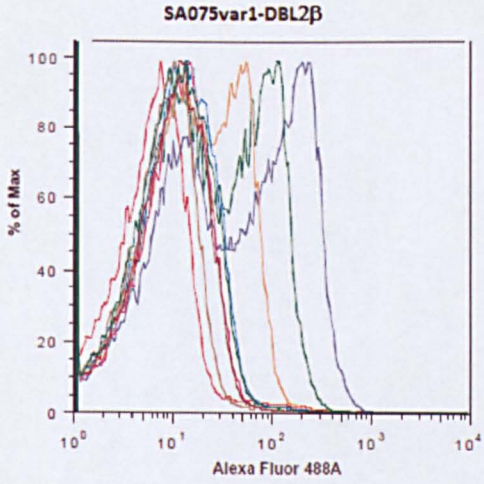
to give a percent of positive cells (Figure 5.3 panel C). The dotted line on the graph represents a cutoff at which 50% of the population remains positive following subtraction of the negative control. Only NTS-DBLα antibodies showed more than 50% positive cells at a concentration of 1.57μg/ml while DBL3γ and DBL4δ showed 50% positivity at 6.25μg/ml. NTS-DBL1α-CIDR1α antibody was at 25μg/ml while CIDR1α and DBL2β antibodies did not show 50% positive cells even at 400μg/ml.

A)


SAO75var1 NTS-DBL1α
SAO75var1 NTS-DBL1α–CIDR–α
SAO75var1-DBL3γ
SAO75var1-DBL4δ
% of Max
Alexa Fluor 488A
KEY
400 µg/ml
100 µg/ml
25 µg/ml
6.25 µg/ml
1.56 µg/ml
0.39 µg/ml
0.09 µg/ml
0.02 µg/ml
400 µg/ml (Rabbit IgG)

B)





KEY

- 400 µg/ml
- 100 µg/ml
- 25 µg/ml
- 6.25 µg/ml
- 1.56 µg/ml
- 0.39 µg/ml
- 0.09 µg/ml
- 0.02 µg/ml
- 400 µg/ml (Rabbit IgG)

C)

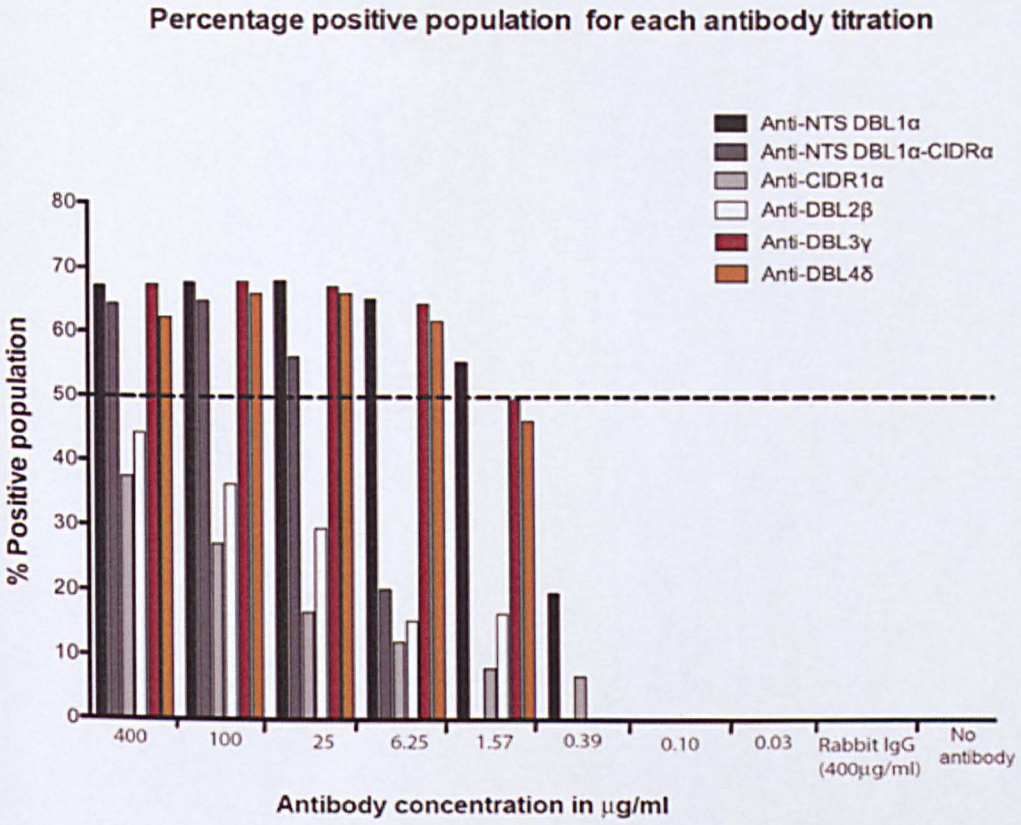


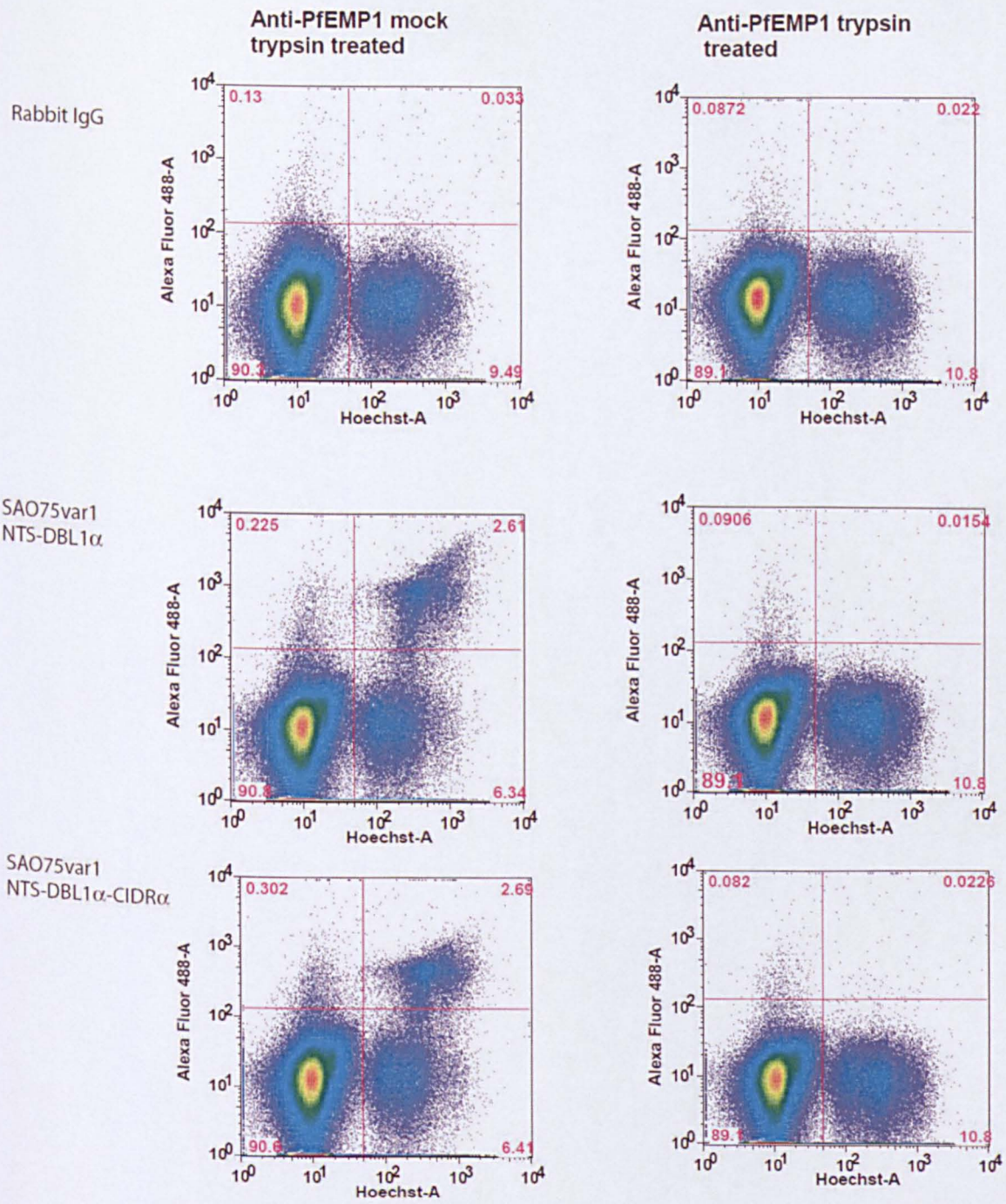
Figure 5.3: Panel A) *Determination of end titre for 4 of the 6 SA075var1 antibodies by flow cytometry. The antibodies against NTS-DBL1α, NTS-DBL1α–CIDR1α, DBL3γ and DBL4δ were diluted four-fold from 400µg/ml down to 0.03 µg/ml and assayed for surface staining against SA075 infected RBCs. Rabbit IgG from a non-immunized rabbit was used as a negative control at a concentration of 400µg/ml. Infected RBCs were stained with Hoechst and surface staining was visualized by a secondary goat anti-rabbit labeled Alexa Fluor 488. The end titre was defined as the lowest concentration of antibody giving surface staining above rabbit IgG background levels.* ***Panel B)*** *2 of the 6 antibodies against CIDR1α and DBL2β analyzed as in* ***A*** *above but on a different day.* ***Panel C)*** *Histograms showing results for determination of end titre by percentage*

positive population. Analysis was done using the Overton cumulative subtraction method in FlowJo. In this analysis, the proportion of negative control i.e. rabbit IgG was subtracted from each sample to give a percentage of positive cells. The dotted line represents a cutoff at which 50% of the population remains positive following subtraction of the negative control. Only NTS-DBLα antibodies showed more than 50% positive cells at a concentration of 1.57μg/ml.

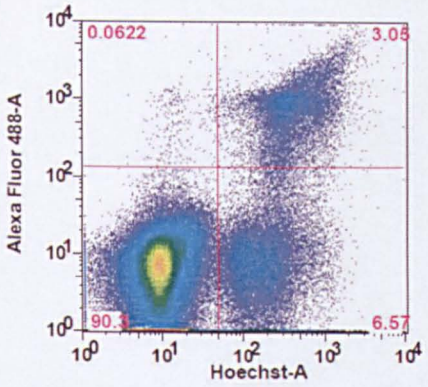
5.4.3: Antibodies against SA075var1 bind to a trypsin-sensitive protein expressed on the surface of mature infected RBCs

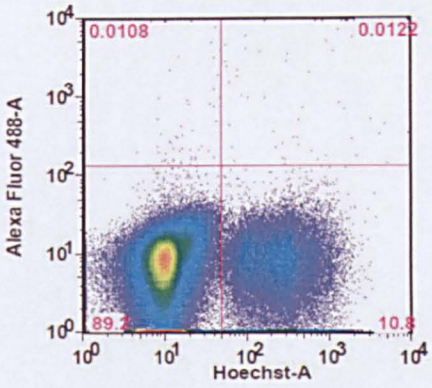
Trypsin sensitivity of *Pf*EMP1 in *P.falciparum* strains has previously been described (Leech, Barnwell et al. 1984; Ghumra, Semblat et al. 2012) and has been used to determine the specificity of anti-*Pf*EMP1 antibody targeting the surface of infected RBCs. Antibodies generated against domains of SA075*var*1 were incubated with trypsin-treated SA075 parasites to determine if they were binding to *Pf*EMP1. Results showed that all the six anti-SA07*var*1 antibodies bound to a trypsin-sensitive protein expressed on the surface of mature SA075 infected cells (Figure 5.4, A and B). This was shown by the abolishment of the double positive-stained population shown on the upper right quadrant of the dotplots for trypsin treated samples compared to the mock-trypsin treated samples. No binding was seen with rabbit IgG control in both cases.

A)

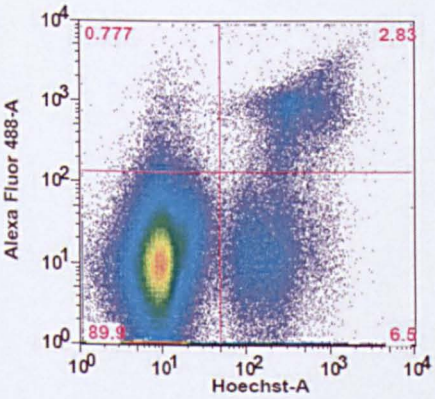

Anti-PfEMP1 mock trypsin treated
Anti-PfEMP1 trypsin treated
Rabbit IgG
SAO75var1 NTS-DBL1α
SAO75var1 NTS-DBL1α-CIDRα
Alexa Fluor 488-A
Hoechst-A
0.13
0.033
9.49
0.0872
0.022
10.8
0.225
2.61
6.34
0.0906
0.0154
10.8
0.302
2.69
6.41
0.082
0.0226

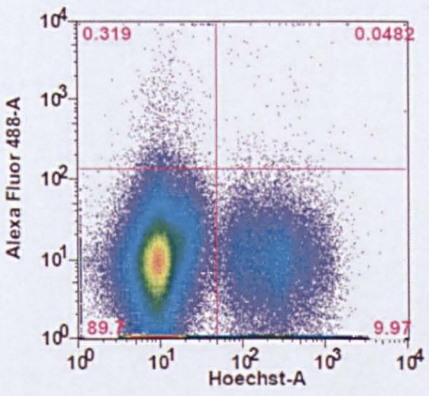
SAO75var1
DBL3γ





SAO75var1
DBL4δ





B)

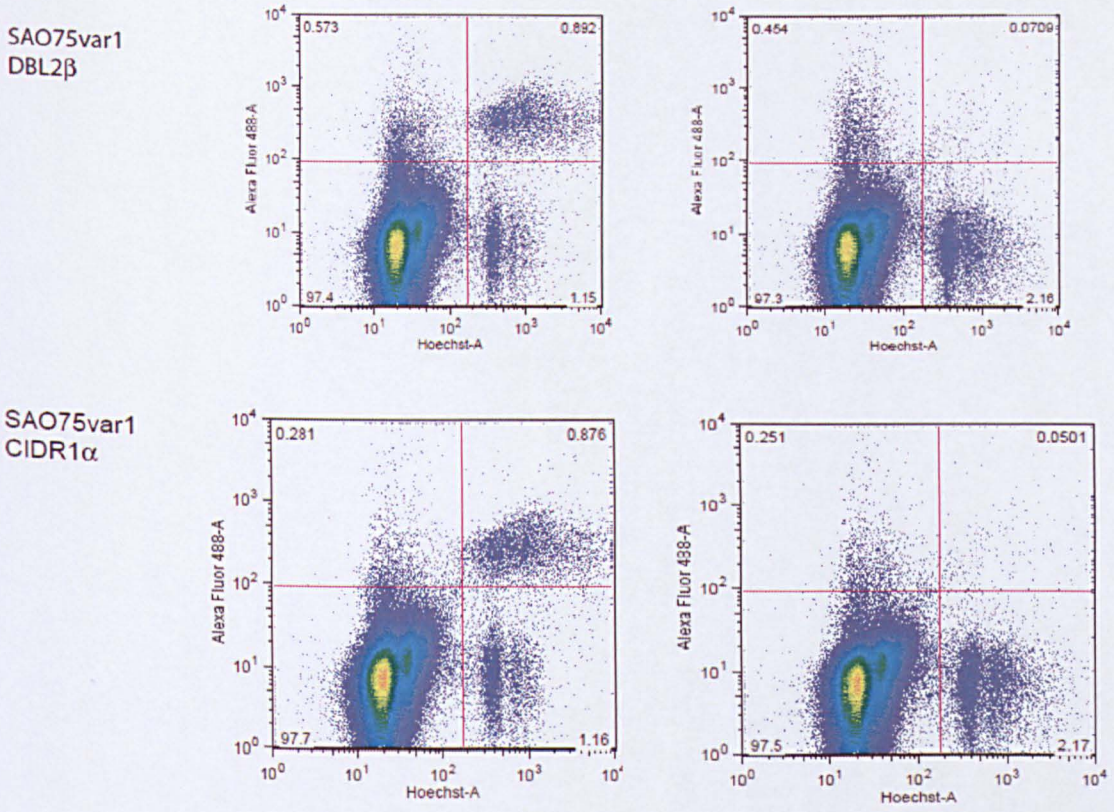


Figure 5.4: A) *Flow cytometry dot plots of trypsin-treated live infected RBCs stained with 400μg/ml of anti-PfEMP1 antibodies against NTS-DBL1α, NTS-DBL1α–CIDR1α, DBL3γ and DBL4δ. SA075 parasites were either mock-trypsin treated (left column) or treated with 10μg/ml of trypsin (right column) for 5 minutes. Both samples were later treated with 1mg/ml of trypsin inhibitor to stop the enzyme reaction. Infected RBCs were stained with Hoechst and anti-PfEMP1 antibodies bound to the surface of infected erythrocytes were detected using Alexa Fluor 488 conjugated to anti-rabbit IgG. The antibodies were shown to target PfEMP1 as evidenced by the lack of staining in the upper right hand quadrant of the trypsin-treated samples compared to the mock-treated samples.* ***B)*** *Flow cytometry dot plots of trypsin-treated live infected RBCs stained with 400μg/ml of anti-PfEMP1*

antibodies against CIDR1α and DBL2β. Assay was carried out as described in A above.

5.4.4: Anti-SA075var1 antibodies mediate phagocytosis of SA075 infected erythrocytes

The ability of antibodies to opsonize and induce clearance of malaria-infected erythrocytes is an important effector mechanism that has been described in both humans and murine models (Shear, Nussenzweig et al. 1979; Celada, Cruchaud et al. 1982). In earlier studies on phagocytosis of malaria parasites, it was shown that the capacity of monocytes and macrophages to phagocytose infected RBCs increased with opsonization of the target with immune sera (Hunter, Winkelstein et al. 1979; Celada, Cruchaud et al. 1982). An *in vitro* phagocytic assay (described in Chapter 2, section 2.3.12) developed by Ataide and colleagues (Ataide, Hasang et al. 2010) was adapted and used to measure antibody-mediated phagocytosis by rabbit generated SA075*var*1 antibodies. However, it is worth noting that while humans have four IgG subclasses, rabbits have only one IgG (Janeway CAJr 1996) and therefore it is possible their effector functions against human infected RBCs may not be directly equivalent. The *in vitro* assay has previously been used to measure phagocytosis mediated by anti-*Pf*EMP1 antibodies in rosetting parasites (Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012). Ethidium bromide-stained infected RBCs were opsonized by antibodies at four concentrations:- 400, 100, 25 and 6.25 μg/ml and incubated with monocytic cells (undifferentiated THP-1), to allow for phagocytosis. Results were analyzed by flow cytometry (FACSCalibur software) in which THP-1 cells were first gated based on FSC and SSC. A second gate was then set on FL3 channel to allow for gating of THP-1 that had phagocytosed infected RBCs and those that had not, as detected by ethidium bromide staining (Figure 5.5). 10,000 of ethidium bromide-positive

THP-1 cells were then acquired at the rate of 200-300 cells /second. Phagocytic activity was normalized against that of a rabbit anti-human erythrocyte antibody (ABCAM ab34858) which was the positive control. Details of the analysis are discussed in Chapter 2, section 2.3.12.

Apart from antibodies to DBL2β and CIDR1α, all other antibodies to SA075*var*1 promoted phagocytosis of the homologous SA075 parasite. NTS-DBL1α showed the strongest phagocytic activity at a concentration of 400μg/ml although anti-DBL3γ was more sensitive at lower concentration of 6.25μg/ml (Figure 5.6). Anti-NTS-DBL1α-CIDR1α caused moderate phagocytosis while anti-DBL4β was effective only at 400μg/ml.

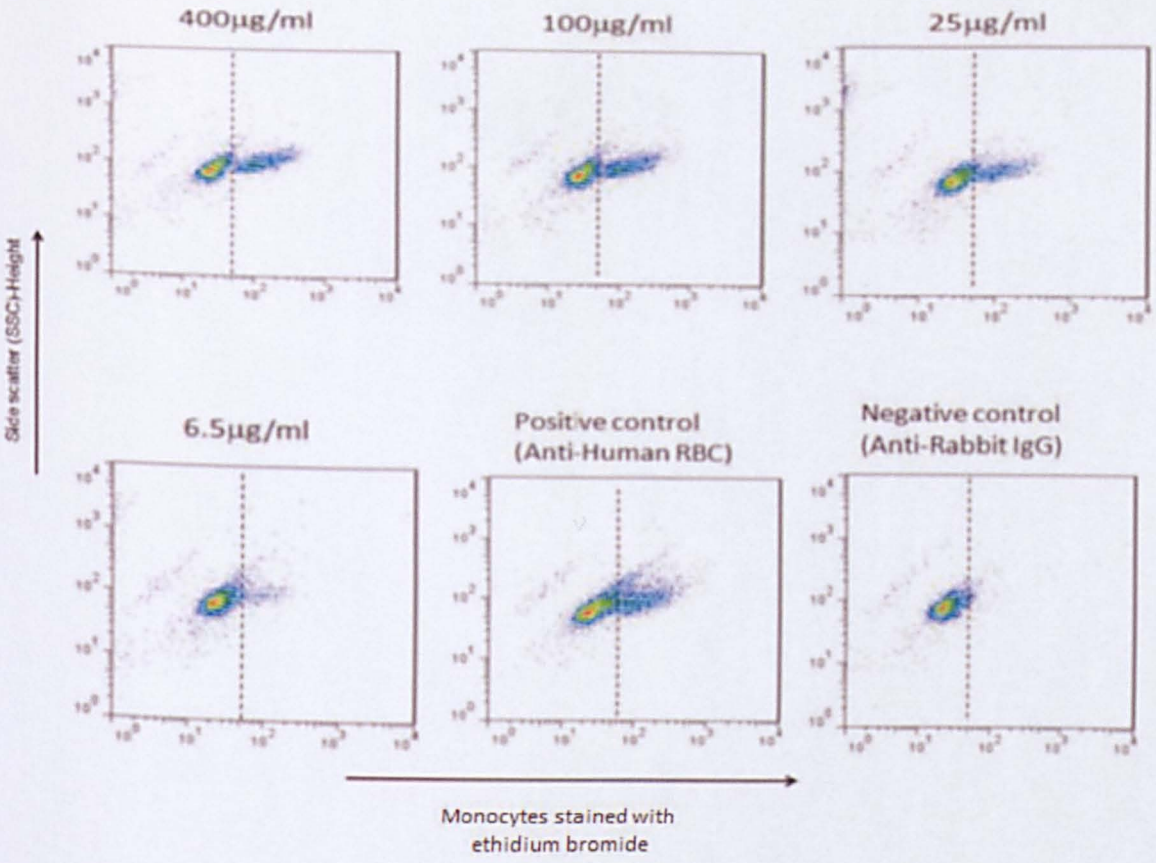


Figure 5.5: *FACs plots for phagocytosis of SA075 infected RBCs by monocytes (THP1 cells). Macs purified infected RBCs were opsonized with four different concentrations of anti-NTS-DBLα antibody. The positive control in this assay was a polyclonal rabbit anti-human RBC IgG antibody used at 90µg/ml and the negative control was anti-rabbit IgG control. The Y-axis (side scatter -SSC) gates the monocytes while the X-axis shows monocytes stained with ethidium bromide on FL3. The dotted line represents the gating for the monocytes that were either ethidium bromide positive or negative.*

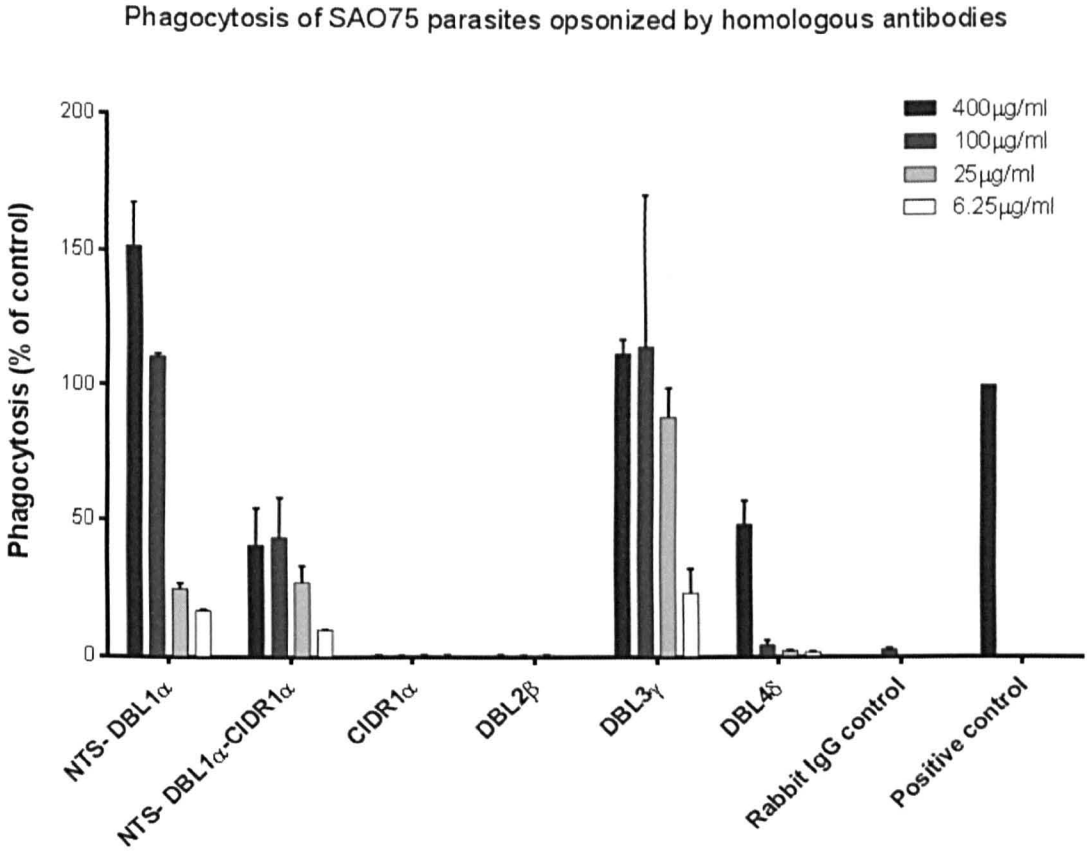


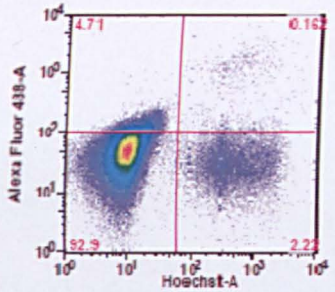
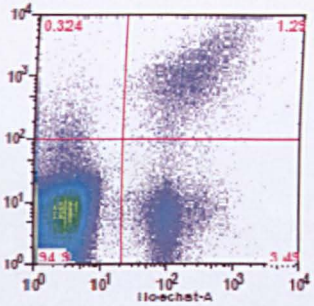
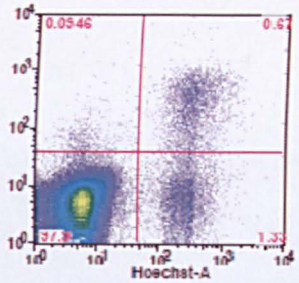
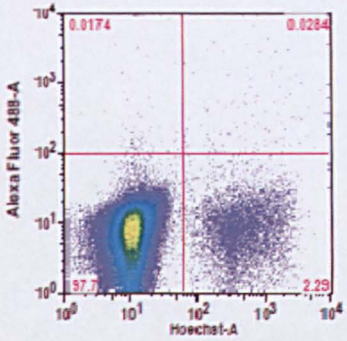
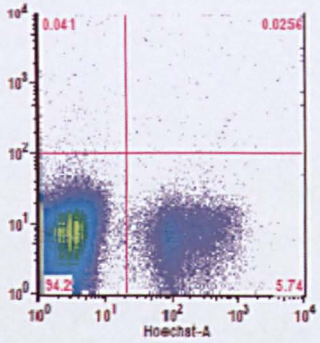
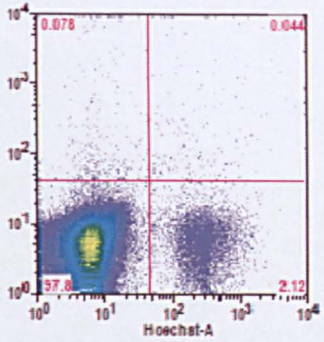
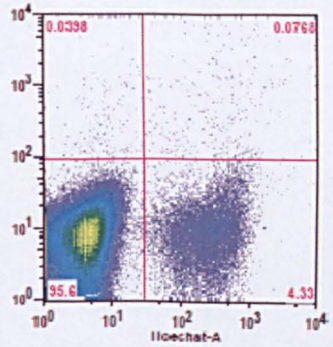
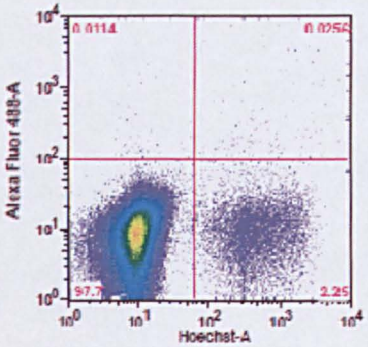
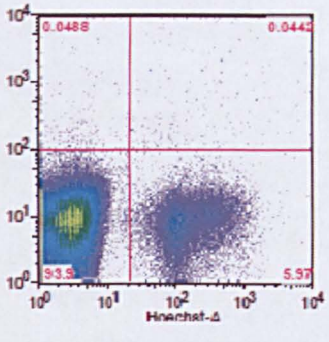
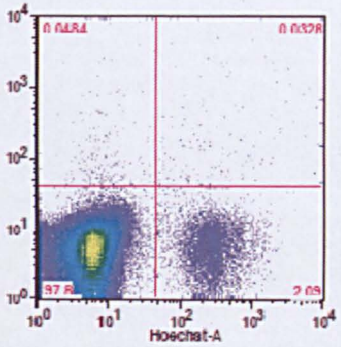
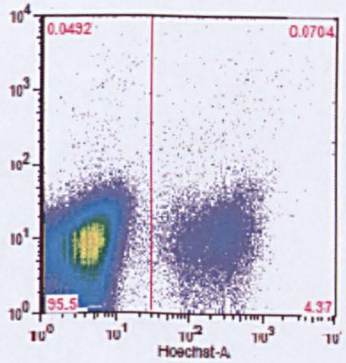
Figure 5.6: *Phagocytosis of SA075 infected erythrocytes by THP-1 cells following opsonization by six anti-SA075var1 antibodies. Rabbit IgG from a non-immunized rabbit was included as a negative control. Data are shown as a percentage of the positive control which was opsonized with a rabbit anti-human erythrocyte antibody.*

5.4.5: Antibodies to SA075var1 domains show an overall strain-specific response but with minimal cross-reactivity

Antigenic relationship of *Pf*EMP1 variants was evaluated by testing variant-specific antibodies against heterologous parasites to check for cross reactivity. A recent study on antibodies to extra-cellular domains of R29 showed a strain specific

response when tested against other rosetting *P. falciparum* lab strains (Ghumra, Khunrae et al. 2011). Using a similar approach, SA075*var*1 antibodies were tested by flow-cytometry for their ability to recognize the surface of RBCs parasitized with heterologous parasite strains (Figure 5.7 and 5.8). The laboratory strains tested were:- R29+, TM180R+, Muz12R+, PAR+, TM284R+, HB3R+ and one culture-adapted field isolate 9197R+. The parasites were grouped into either IgM or non-IgM binders (Table 5.1.). Antibodies were tested at a concentration of 400 µg/ml. Although generally strain-specific, two of the SA075*var*1 antibodies i.e. anti-CIDR1α, anti-DBL2β cross-reacted with TM284R+, an IgM-binding parasite (Figure 5.8B and summary table 5.1).

Cross reactivity of SA075 antibodies against non-IgM binding parasites

Antibodies to:-	MUZ12	R29	TM180	9197
Homologous NTS-DBL1α				ND
SAU/Svar1 NTS-DBL1α				
SAU/Svar1 NTS-DBL1α–CIDR1α				

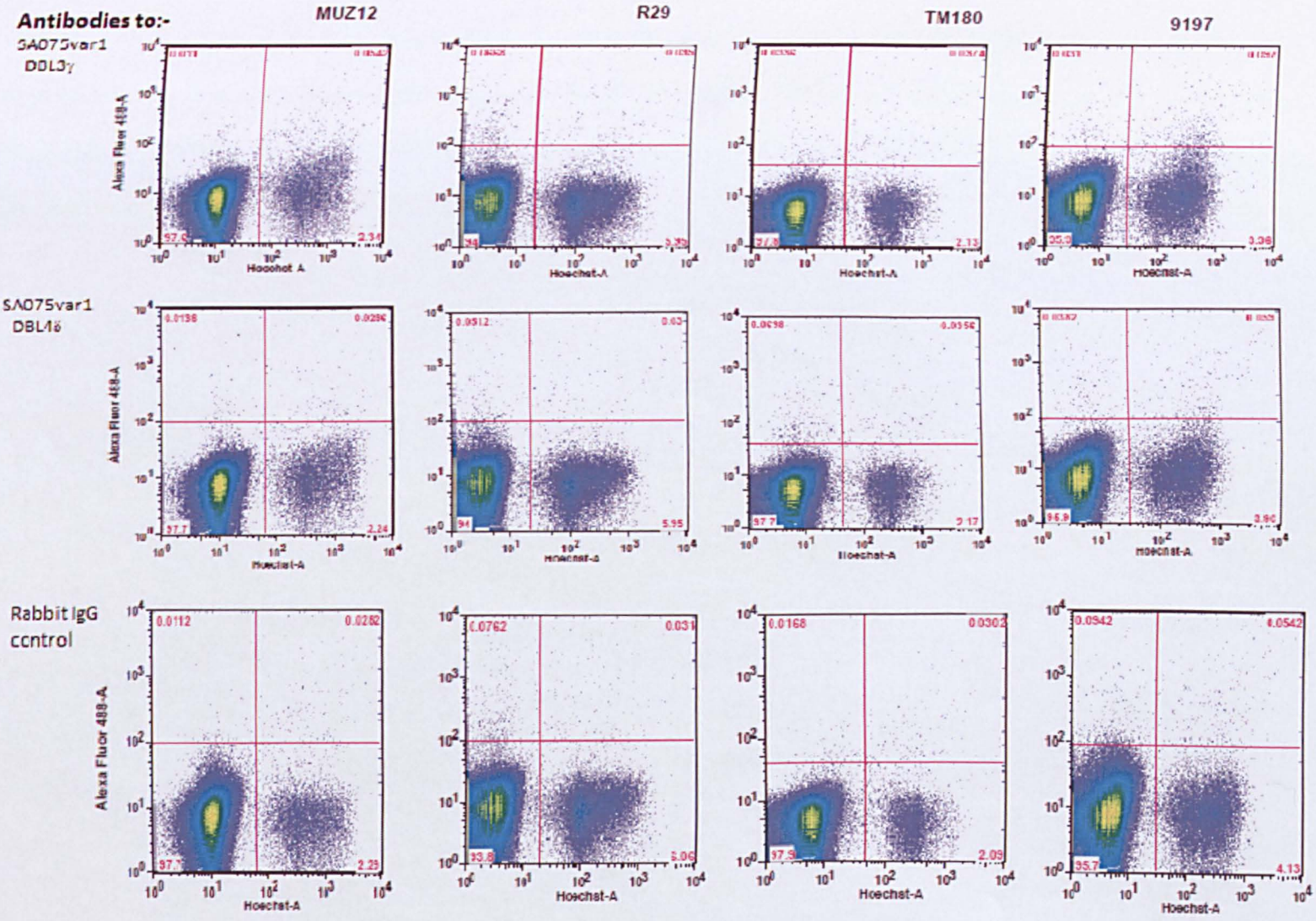
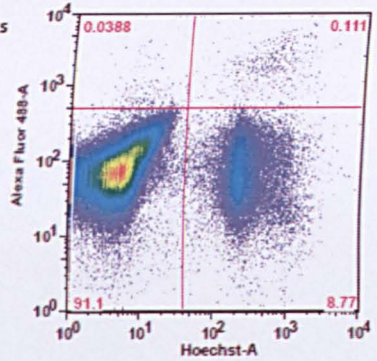
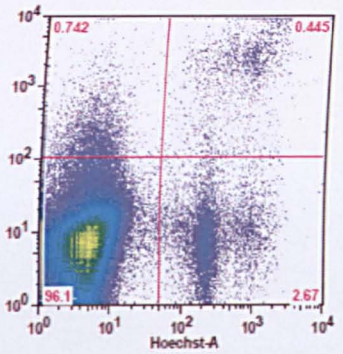
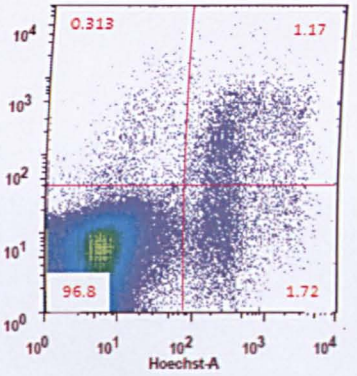
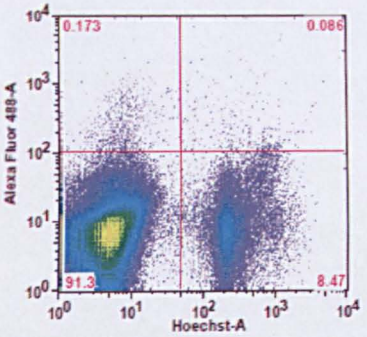
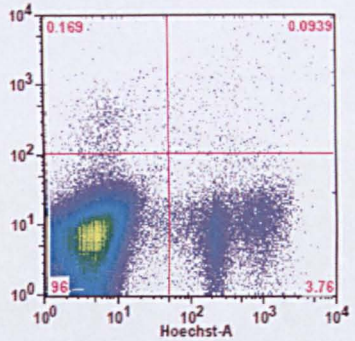
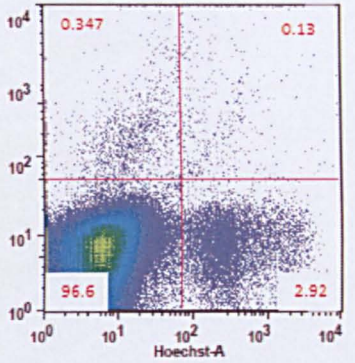
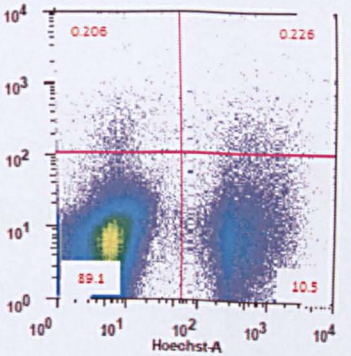
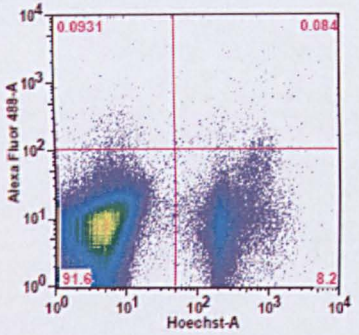
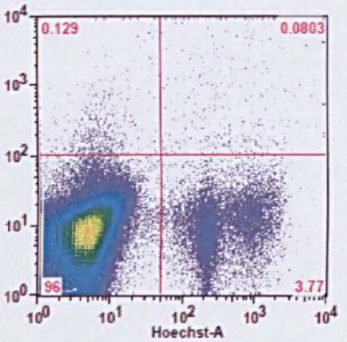
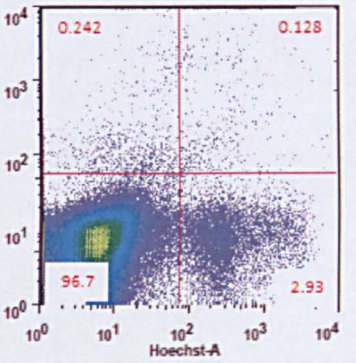
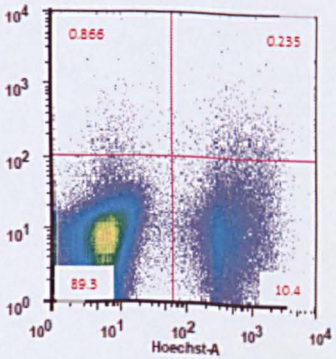

Antibodies to:-
MUZ12
R29
TM180
9197
SA075var1 DBL3γ
SA075var1 DBL4δ
Rabbit IgG control
Alexa Fluor 488-A
Hoechst-A

Figure 5.7A: *Flow cytometry dot plots showing cross reactivity of anti-SA075var1 antibodies against non-IgM binding parasites (Muz12R+, R29R+, TM180R+ and a recently culture-adapted field isolate 9197R+ :-Homologous NTS-DBL1α for each isolate (except 9197) followed by antibodies against SA075 var1-NTS-DBL1α, NTS-DBL1α–CIDR1α, DBL3γ and DBL4δ and rabbit IgG control antibody. Infected RBCs were stained with Hoechst and anti-PfEMP1 antibodies bound to the surface of infected erythrocytes were detected using Alexa Fluor 488 conjugated to anti-rabbit IgG.*

Antibodies to:-	MUZ12	R29	TM180	9197
Homologous NTS-DBL1α				Not done
SA075var1 DBL2β				
SA075var1 CIDR1α				

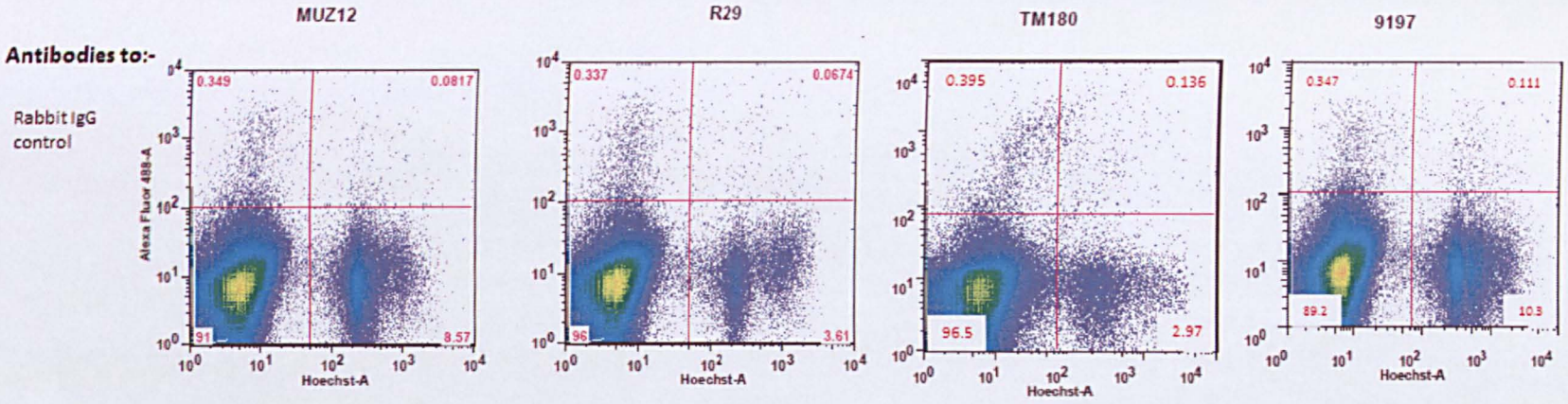
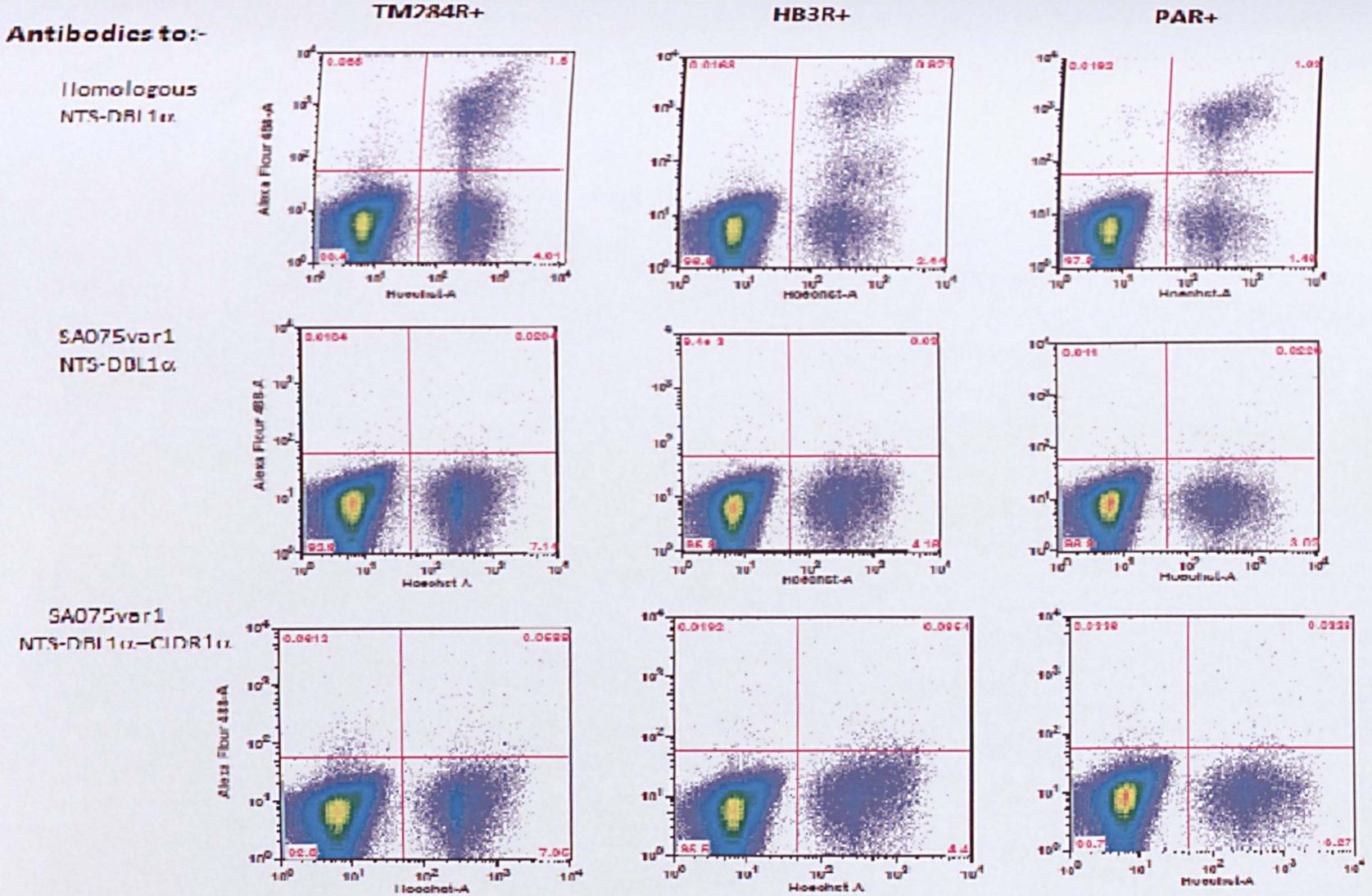


***Figure 5.7: B**) CIDR1α, DBL2β and rabbit IgG control antibody tested in a similar manner as **A** above. Infected RBCs were stained with Hoechst and anti-PfEMP1 antibodies bound to the surface of infected erythrocytes were detected using Alexa Fluor 488 conjugated to anti-rabbit IgG.*

5.8 A: Cross reactivity of SA075 antibodies against IgM binding parasites



Antibodies to:-

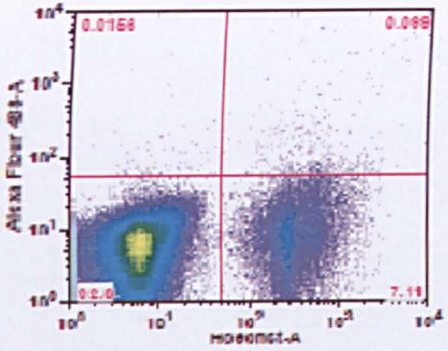
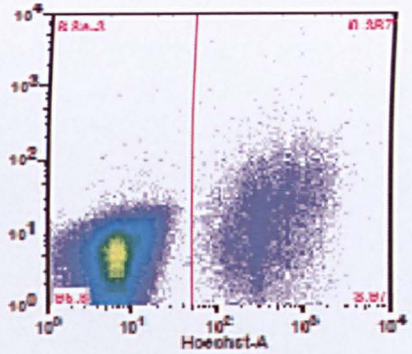
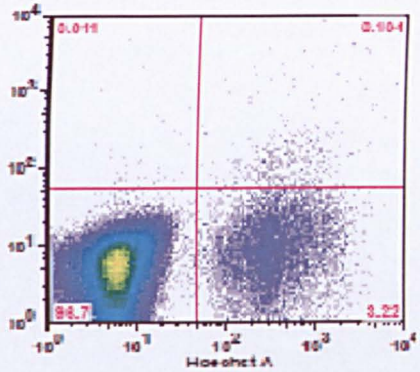
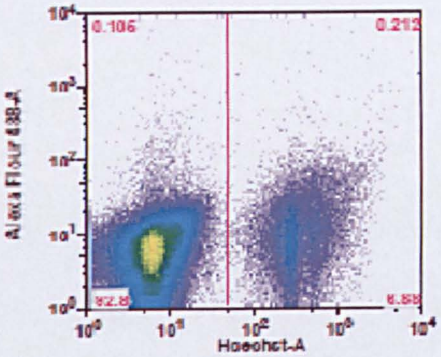
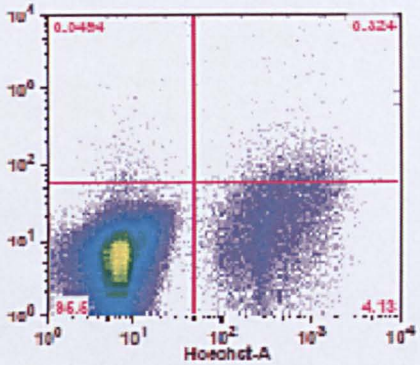
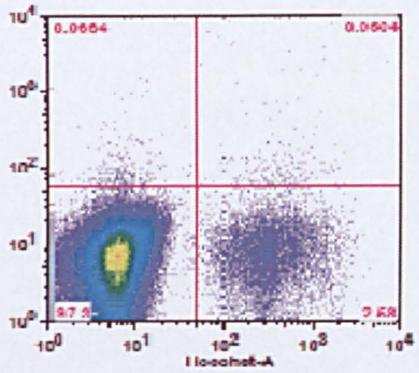
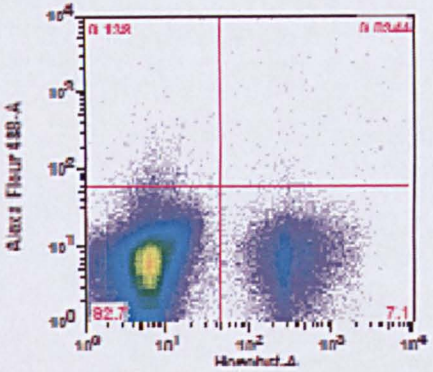
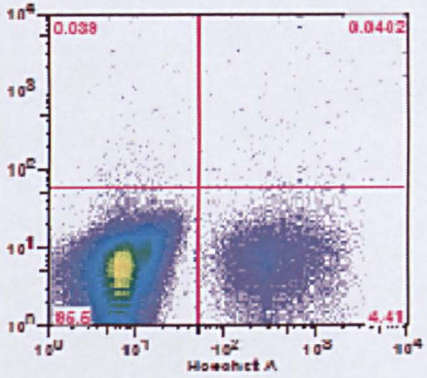
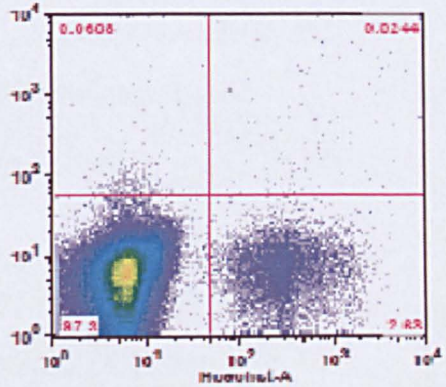
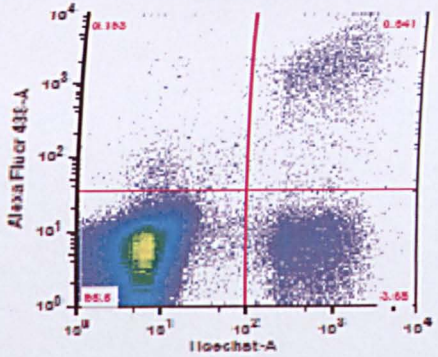
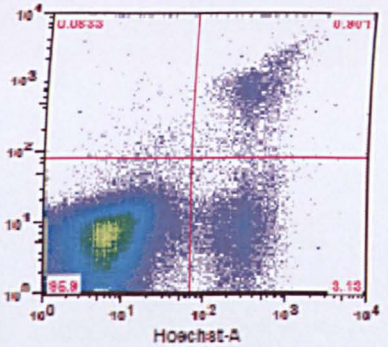
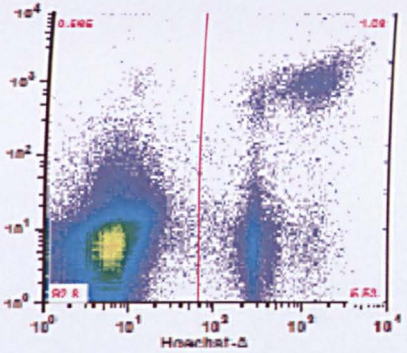
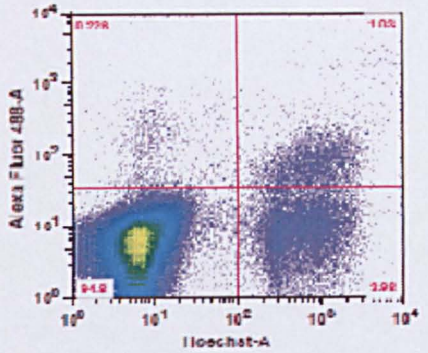
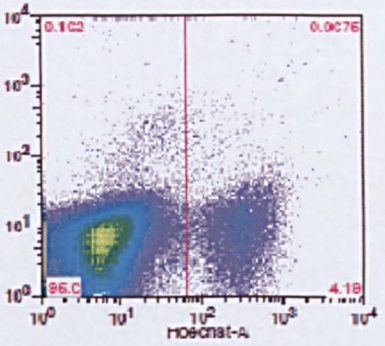
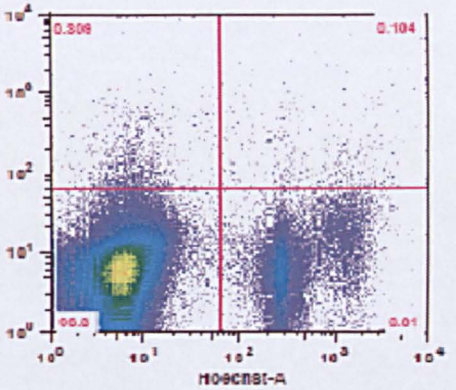
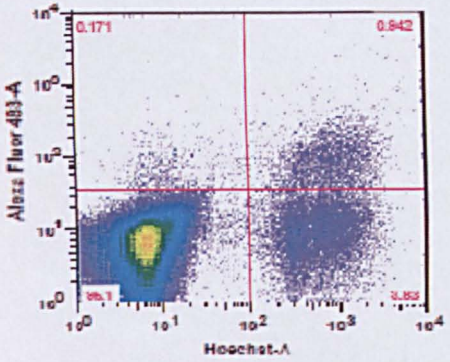
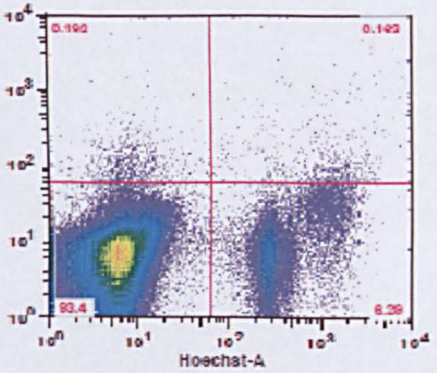
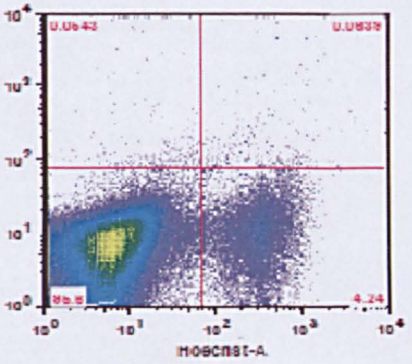
	TM284R+	HB3R+	PAR+
SA075var1 DBL3γ			
SA075var1 DBL4δ			
Rabbit IgG control			

Figure 5.8 A: *Flow cytometry dot plots showing cross reactivity of anti-SA075var1 antibodies against IgM binding parasites (TM284R+, HB3R+ and PAR+) The antibodies used were :-Homologous NTS-DBL1α for each isolate followed by antibodies against SA075 var1-NTS-DBL1α, NTS-DBL1α-CIDR1α, DBL3γ and DBL4δ. Infected RBCs were stained with Hoechst and anti-PfEMP1 antibodies bound to the surface of infected erythrocytes were detected using Alexa Fluor 488 conjugated to anti-rabbit IgG.*

Antibodies to:-

	TM284R+	HB3R+	PAR+
Homologous NTS DBL1α			
SA075var1 DBL2β			
SA075var1 CIDR1α			

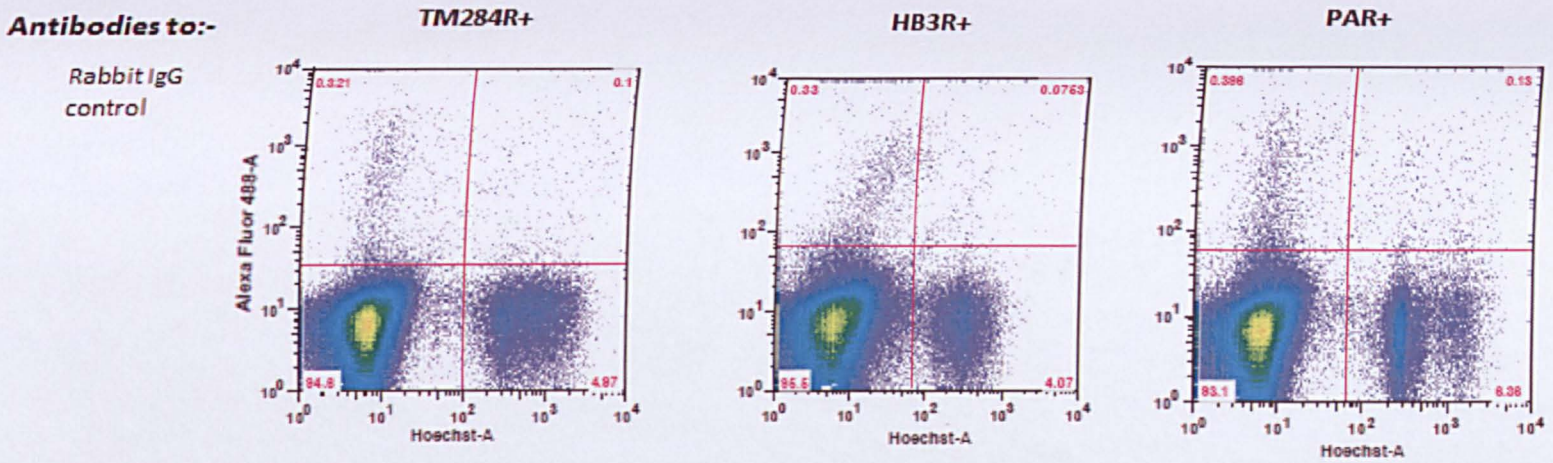
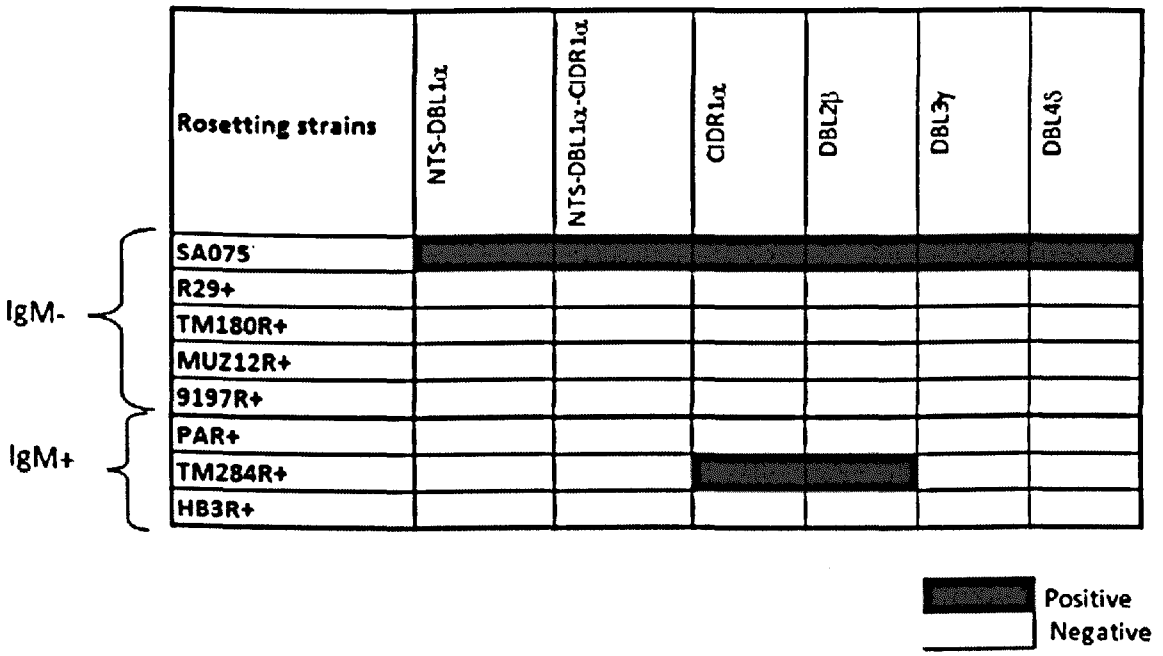


Figure 5.8B: *Flow cytometry dot plots showing cross reactivity of anti-SA075var1 antibodies against IgM binding parasites (TM284R+, HB3R+ and PAR+).The antibodies used were CIDR1α and DBL2β and rabbit IgG control tested in a similar manner as* ***A*** *above. Infected RBCs were stained with Hoechst and anti-PfEMP1 antibodies bound to the surface of infected erythrocytes were detected using Alexa Fluor 488 conjugated to anti-rabbit IgG.*

Table 5.1: *A summary of cross reactivity of anti-SA075 antibodies against other rosetting parasites as determined by FACS. As previously described in section 5.4.2, a positive value was defined as the lowest concentration giving a positive signal above the rabbit IgG control. The first row represents recognition of SA075 parasites, all of which were positive. The only cross reactivity seen was by anti-CIDR1α and anti-DBL2β against TM284, an IgM binding parasite. The ability of each rosetting type to bind non-immune IgM is indicated on the left as either IgM- or IgM+.*



5.4.6: Use of Anti-NTS-DBLα antibody for cell sorting and correlation of FACS generated % positive population with rosetting frequency

It is possible that there can be several *var* genes that are involved in rosetting in a haploid genome. An example is in the IT genome where IT*var*9 and IT*var*60 have been shown to be involved in rosetting in different parasite clones (Rowe, Moulds et al. 1997; Albrecht, Moll et al. 2011). Unfortunately in *in vitro* culture, this may result in a heterogenous population in which rosettes in a single culture are mediated by different *Pf*EMP1 variants. A study by Vigan-Womas *et al* 2008 (Vigan-Womas, Guillotte et al. 2008) showed how a heterogenous population of *Var* O rosetting parasites resulted in discrepancy between the rosetting frequency by microscopy and % positive population by FACS. However, following panning by an anti-*Var*O monoclonal antibody, there was a high correlation between the two reads. It was therefore important that a homogenous population of SA075 expressing SA075*var*1 be maintained in continuous culture. A cell sorting protocol (described in section 5.3.2) was developed that allowed for selection of a monovariant-enriched SA075 parasites using anti-NTS-DBL1α antibody. Once optimized, the protocol was later adapted for use on other *P.falciparum* lab strains using their respective homologous antibodies. All the cell-sorted parasites were frozen down and later used to examine clinical relevance of rosetting variants using plasma from previously sick children (Chapter 6). Figure 5.9 shows an example of 1) unsorted versus 2) cell-sorted SA075 parasites stained with rabbit IgG control (left panel) and anti-NTS-DBLα antibodies (right panel). The percentage of the positive population is indicated on the top right quadrant. Results show that the cell sorting protocol was effective by causing a 50% increase in the positive population.

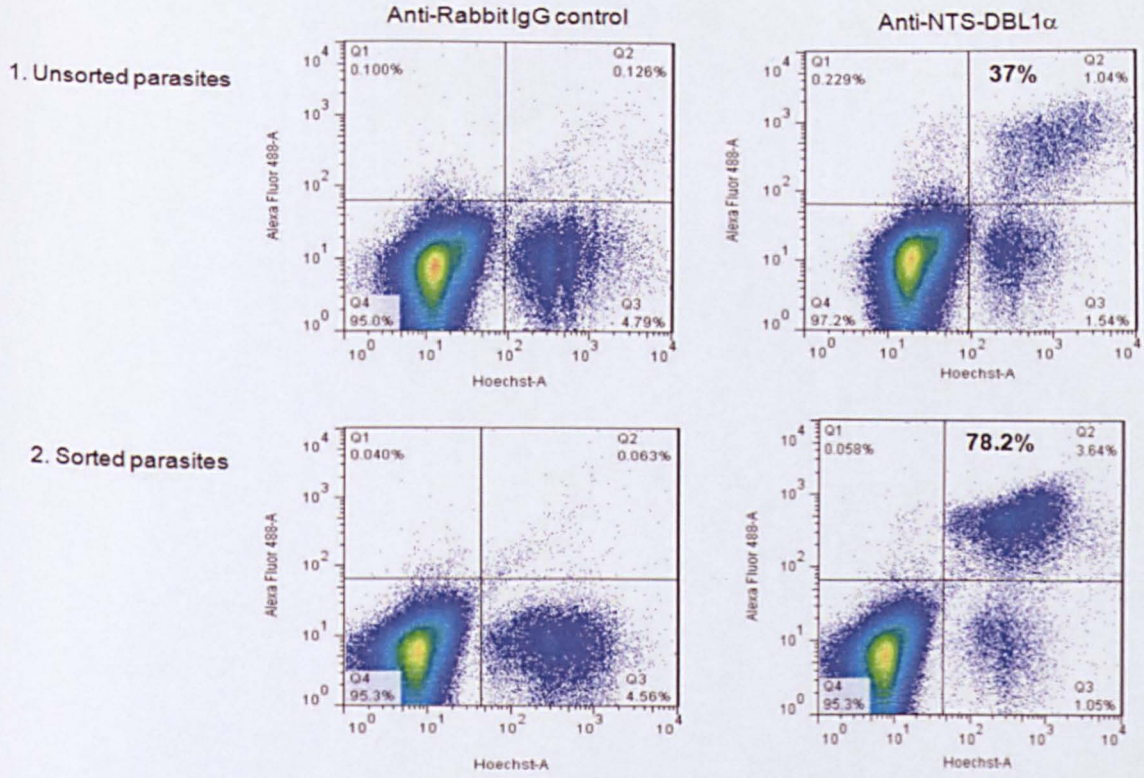


Figure 5.9: *FACs plots showing staining SA075 parasites before (top right) and after (bottom right) cell sorting. In each assay, a rabbit IgG control was included. The proportion of cells positive for NTS-DBLα antibody staining was 37 % in the unsorted population and increased to 78.2 % in the sorted population.*

Does the % positive population correlate with rosetting frequency?

It was important to determine how the % positive population by FACS assay compared to the rosetting frequency (% RF) by microscopy. An ideal situation would be a positive correlation between the two reads thus supporting identification of SA075*var*1 as the rosette mediating *Pf*EMP1 variant. A poor/negative correlation on the other hand would indicate that the *Pf*EMP1 being enriched was probably not responsible for rosetting. A systematic assay was therefore set up to compare the % positive population by FACS and the % RF by microscopy for both the cell sorted and unsorted SA075 parasites (summary in

Table 5.2). Microscopy reads were made by two independent readers. Results showed inconsistencies between the % positive population by FACS and rosetting frequencies in both the unsorted and the cell sorted parasite populations (Table 5.2). First, the unsorted parasites showed a discrepancy between microscopy and FACS with a % RF (average score of 21%) and % positive population (51%) respectively. One round of cell sorting however resulted into parallel reads where an average % RF of 38% and % positive population of 32% were observed. It is not clear why there was a drop in % positive population from the original 51% to 32%. However, in an attempt to increase the rosetting frequencies, two rounds of rosette selection by Percoll flotation method (Chapter 2, section 2.3.4) were later done on the cell-sorted parasite and this resulted in a higher average % RF (66.4%) that correlated well with the % positive population by FACs (69%). These results were encouraging and suggested that SA075*var*1 was indeed responsible for rosetting in SA075 parasites. However, a further attempt to increase the homogeneity of the sorted parasites by a second round of sorting resulted into an even bigger discrepancy between % RF (average 31%) and % positive population (72.9%). Table 5.2 summarizes the data comparing % RF by microscopy and % positive population by FACS.

Table 5.2: *A comparison between rosetting frequency by microscopy and % positive population by FACS in unsorted and cell sorted SA075 parasites*

	Parasite	%Rosetting frequency by microscopy		% positive population by FACs
		1st Reader	2nd Reader	
1	SA075 Unsorted	17	25	51
2	SA075_First round cell sorting	42	35	32
3	SA075_Cell sorted once with two rounds of Percoll selection.	62.7	70	69
4	SA075_Second round cell sorting	24	38	72.9

5.5: DISCUSSION

This chapter focused on functional characterization of six anti-*Pf*EMP1 antibodies targeting extra-cellular domains encoded by putative rosette mediating *var* gene from a field isolate from Kisumu, Kenya. One key finding in this chapter was the possibility that SA075*var*1 *Pf*EMP1 variant may not be involved in rosetting in SA075 parasites. In a comparison of rosetting frequency (% RF) by microscopy and the % positive population by FACs assay on both the unsorted and cell sorted SA075 parasites (Table 5.2), inconsistent results were observed that could suggest that SA075*var*1 was not the rosette mediating variant in SA075 parasites. It would be expected that if SA075*var* 1 was responsible for rosetting in SA075, the RF by microscopy would closely match the % positive population by FACs stained by an anti-SA075*var*1 antibody. In some instances however, the % RF was found to be lower than the % positive population by FACS. A possible explanation would be that the rosettes in SA075 are generally "weak" and that any slight disruption would result in lower % RF counts compared to the positive

population by FACs staining. It could also be that SA075*var*1 is indeed responsible for rosetting but the anti-SA075*var*1 antibodies cross-react with different variant within the SA075 genome resulting into high % positivity by FACS and low rosetting frequencies. This may however be ruled out by the fact that all the six SA075*var*1 antibodies to different domains recognized the same infected RBC population in multiple FACS experiments hence it is unlikely there is cross-reactivity with a different variant.

None the less, the SA075*var*1 antibodies were tested for their ability to recognize *Pf*EMP1 on the surface of live infected RBCs and mediate phagocytosis. One of the antibodies was further used to develop a cell sorting protocol that was later adapted and used to select for monovariant-enriched parasites in other *P.falciparum* strains.

It is known that acquired protective immunity to *malaria* infection relies on antibodies recognizing the variant surface antigens such as *Pf*EMP1, expressed on the surface of late-stage-infected RBCs (Marsh and Howard 1986; Bull, Lowe et al. 1998). Antibodies to rosette mediating-*Pf*EMP1 variants, and particularly those from NTS-DBLα domain, have been widely examined in immunological studies. Only one study by Ghumra *et al.*, 2011 (Ghumra, Khunrae et al. 2011) has so far done a systematic characterization of antibodies to recombinant extracellular domains of a rosetting *Pf*EMP1 variant i.e. IT*var*9. In this chapter, the specificity of six SA075*var*1 antibodies binding to live infected RBCs was evaluated by IFA and FACS assays (Figure 5.2). All antibodies to the five domains and one di-domain of SA075*var*1 recognised the native protein on the surface of live infected RBCs with the IFA results showing a punctuate fluorescent staining pattern that is typical of *Pf*EMP1 as previously described (Baruch, Pasloske et al.

1995; Ghumra, Semblat et al. 2008; Ghumra, Khunrae et al. 2011). A further confirmation that the antibodies were targeting *Pf*EMP1 on the surface of infected SA075 RBCs was in the trypsin experiment (Figure 5.4), where all the antibodies showed absence of binding to the trypsin-treated cells, indicating that the trypsin sensitive *Pf*EMP1 had been abolished. Although all the antibodies recognized the surface of infected RBCs, there was a difference in the levels of binding sensitivities or end titres. NTS-DBLα showed the highest sensitivity, reacting with >50% parasite positive population at a concentration of 1.57μg/ml (Figure 5.3 A and C). The high sensitivity could perhaps be because the domain is more exposed in the final protein than the other domains or might simply reflect higher affinity antibodies due to a higher quality recombinant protein generation. On the other hand, there was low binding sensitivity by anti-CIDR1α and anti-DBL2β antibodies (Figure 5.3 B and C). This could be attributed to several factors: -1) The two domains, which occur next to each other in tandem are perhaps not fully exposed to the surface to allow for antibody binding. 2) It could also be that the proteins used for immunization did not contain correct conformational epitopes hence the low reactivity of these two antibodies. For both CIDRα and DBLβ there was very little protein at the predicted molecular weight as compared to proteins from the other domains (Figure 4.11). It would be interesting to find out whether the two domains are recognized by antibodies in ELISA, although this does not often correlate well with surface recognition (Vigan-Womas, Guillotte et al. 2011; Ghumra, Semblat et al. 2012). A previous field study done by Mackintosh and colleagues (Mackintosh, Christodoulou et al. 2008) to examine the acquisition of naturally acquired antibodies to recombinant domains of A4U parasites showed evidence for age-associated acquisition of domain-specific IgG for all the domains, except CIDR1α and DBL2β (Mackintosh, Christodoulou et al. 2008) by ELISA. In

the same study, antibody responses to recombinant CIDR1α domain were significantly negatively correlated with those of intact A4U parasites. Although this is not indicative of conservation of CIDR1α and DBL2β sequences in SA075*var*1 and A4*var*, it may support the idea that some *Pf*EMP1 domains may be generally more exposed than others in the overall *Pf*EMP1 protein structure.

SA075*var*1 antibodies were tested for their ability to mediate phagocytosis. This effector mechanism is thought to be important for antibodies that recognize the surface of infected RBCs (Langhorne, Ndungu et al. 2008). In antibody mediated phagocytosis assay, only four of the six antibodies were able to induce phacytosis when opsonized with SA075 infected RBCs. Anti-CIDR1α and anti-DBL2β did not induce phagocytosis even at the highest antibody concentration of 400μg/ml. Note that the two antibodies also had the lowest end titres when tested for surface recognition. It could be that induction of phagocytosis requires not only a certain threshold concentration, but also antibodies of a certain quality. One caveat in this assay could be in the use of rabbit generated antibodies against human RBCs. It is possible that the mechanism of antibody-mediated phagocytosis are different in humans versus rabbits considering that rabbits have only one IgG class while humans have 4 IgG subclasses, two (IgG1 and IgG3) of which are more efficient in mediating phagocytosis (Tebo, Kremsner et al. 2001; Tebo, Kremsner et al. 2002).

To investigate the antigenic relationship of different rosetting parasites, SA075*var*1 antibodies were used in surface staining assays against seven heterologous parasites from different geographical locations and with different IgM binding phenotypes. Apart from anti-CIDR1α and anti-DBL2β which cross-reacted with TM284R+ parasites, all other antibodies showed a variant specific-response.

Interestingly, these are the two antibodies that showed weak surface recognition and inability to induce phagocytosis. The cross reactivity by these two antibodies was rather surprising because a recent study by Ghumra *et al.*, 2012 (Ghumra, Semblat et al. 2012) showed induction of strain transcending antibodies that seemed to be limited to rosetting parasites with IgM binding phenotype. This was not the case with antibodies from the group of parasites with non-IgM binding phenotype to which SA075 parasites belongs, discussed in chapter 4, section 4.4.4. However, the antibodies tested in the Ghumra 2012 study (Ghumra, Semblat et al. 2012) were all from the NTS-DBLα domain and therefore the role of antibodies to the downstream domains of *Pf*EMP1 variants remains unknown. At the sequence level, TM284*var*1 and SA075*var*1 belong to different CP groups, different block sharing (BS) groups (Chapter 4, Table 4.6) and have different domain architectures (Chapter 4 Figure 4.10). They also posses different domain cassettes i.e. DC11 and DC8-like respectively. The cause of the cross-reactivity by antibodies to the two domains therefore remains unknown.

Antigenic switching is one major caveat in the study of *var* genes and their association with particular adhesion phenotypes. In rosetting parasites, this could result in a heterogenous population of rosette-forming parasites expressing different *Pf*EMP1 variants. The ability of SA075*var*1 antibodies to react with the surface of live infected RBCs was exploited in a cell sorting FACS assay, in which anti-NTS-DBL1α antibody was used to isolate parasites expressing SA075*var*1. The protocol was later adopted and used for all other *P.falciparum* strains which were used in examining the clinical relevance of rosetting variants as will be discussed in chapter 6. A similar strategy has been used for *Var*O expressing

parasites although the technique used was panning using a specific monoclonal antibody (Vigan-Womas, Guillotte et al. 2008) and not cell sorting.

In conclusion, in spite of the possibility that SA075*var*1 does not mediate rosetting in SA075, this study confirms previous observations that it is possible to induce functional antibodies from *Pf*EMP1 variants. Antibodies raised against NTS-DBL1α domains have shown greater surface reactivity, sensitivity and functional activity such as promoting phagocytosis of homologous parasite strains compared to antibodies raised against domains downstream of NTS-DBL1α. Here, we also confirm that antibodies to non-IgM binding parasites generally induce strain specific responses although 2 out of 6 of the antibodies showed cross reactivity with TM284R+, a parasite with IgM-binding phenotype. More rosette-mediating variants especially from field isolates will need to be studied to fully understand the molecular mechanisms of rosetting and the importance of anti–rosetting antibodies.

CHAPTER 6

Clinical relevance of rosette-mediating *Pf*EMP1 variants

6.1: INTRODUCTION

6.1.1:Immunity against rosette forming parasites

Natural immunity to malaria in endemic areas is generally acquired after repeated exposure to infection (Marsh and Kinyanjui 2006; Doolan, Dobano et al. 2009). It is believed that immunity to variant surface antigens (VSAs) develops in a similar manner in which variant-specific responses develop against each VSA that the host encounters (Ofori, Dodoo et al. 2002). Following cumulative exposure to multiple parasite infections over time, a broad repertoire of strain specific immune responses then develops that can recognize different VSAs (Doolan, Dobano et al. 2009). This kind of build-up in immunity has been reported for most of the surface antigens presented during the *Plasmodium falciparum* asexual cycle. As discussed in Chapter 1 section 1.7, the VSA described here will refer mainly to *Plasmodium falciparum* erythrocyte membrane Protein 1 (*Pf*EMP1).

Studies on antibody responses against rosetting strains have shown the presence of anti-*Pf*EMP1 antibodies and that the potential protective mechanisms of these antibodies includes anti-rosetting activity (Carlson, Helmby et al. 1990; Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012) and their ability to mediate phagocytosis (Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012). Antibody responses by human plasma samples against recombinant domains of rosetting parasites (and particularly NTS-DBLα) have previously been measured by ELISA (Mayor, Rovira-Vallbona et al. 2009; Vigan-Womas, Lokossou et al. 2010). These results suggest that despite the extensive polymorphisms,

individuals do raise antibodies against recombinant domains of rosette-*Pf*EMP1 variants. However, one possible caveat with the use of recombinant proteins may be the improper folding of the proteins which could result in either absence of binding or differences in the recognition of linear epitopes compared to conformational epitopes, a concern that has been previously discussed (Vigan-Womas, Guillotte et al. 2011; Ghumra, Semblat et al. 2012) (Angeletti, Albrecht et al. 2013). Recognition of the native *Pf*EMP1 on the surface of live infected RBCs by anti-*Pf*EMP1 antibodies (Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012) has therefore been an alternative approach. Analyzing immune responses against rosette-mediating variants may help us to understand the link between rosetting and severe disease and how this immunity protects against severe disease. The importance of such studies is that despite the consistent association between rosetting and severe malaria in Sub-Saharan Africa (Carlson, Helmby et al. 1990; Rowe, Obeiro et al. 1995; Newbold, Warn et al. 1997; Kun, Schmidt-Ott et al. 1998), the exact mechanisms by which rosettes cause severe disease remain unclear.

An ideal way to directly link rosetting to pathogenesis of severe disease would be to use an animal model in which the entire process of development of severe disease due to rosetting and the acquisition of antibodies would be monitored. However, such models as reviewed by Craig et al., 2012 (Craig, Grau et al. 2012) are not able to reproduce the spectrum of syndromes and protection that is seen in humans. This chapter focuses on examining the *in-vivo* relevance of rosette-mediating variants by studying the antibody responses to cell sorted rosetting parasites. Using patient's plasma samples taken at the time of disease (acute) and 2-4 weeks later (convalescent), the kinetics of variant-specific antibody responses were studied in patients with different malaria clinical syndromes compared to

uncomplicated malaria controls. An increase in antibody levels between the two time points would suggest that the rosetting variant being tested was antigenically similar to the variant/s expressed by the parasite at the time of disease thus linking the variant to the particular syndrome.

6.2: CHAPTER AIMS

The main aim of this chapter was to study variant-specific antibody responses against rosette-mediating variants and determine whether certain *Pf*EMP1 variants could have played a role in particular disease outcomes. The newly described SA075*var*1 variant (chapter 4) was also included. Using acute-convalescent plasma pairs from carefully matched malaria cases and controls, the kinetics of the antibody responses against parasites enriched for single variants were compared. Analysis was also done on unmatched samples in a bigger study. The results would reveal whether particular rosette-mediating variants were antigenically related to parasites that the patients were exposed to at the time of disease.

2.1 Specific aims

i. To compare acute and convalescent antibody responses to different rosetting parasites using plasma from Kenyan children who presented with different clinical syndromes of severe malaria.

ii. As a follow-up to a study by Ghumra *et al.*, 2012 (Ghumra, Semblat et al. 2012), to compare antibody responses to rosetting parasites with IgM binding phenotype versus non-IgM binders.

iii. To study the kinetics of antibody responses to rosette-mediating variants over time.

iv. To examine the relationship between the antibody responses to rosetting variants with rosetting frequency, parasitaemia and age.

v. To compare acute-convalescent antibody responses within samples that had rosetting scores and to correlate them with rosetting frequency.

6.3: MATERIALS AND METHODS

6.3.1: Parasites used in the analysis

Cell-sorted rosetting parasites were used for surface recognition assays in this chapter. The process of cell sorting to select for rosetting parasites enriched for a single variant was done as described in Chapter 5, section 5.3.2 using facilities at the University of Edinburgh, Scotland. Four different laboratory rosetting strains and the recently culture-adapted SA075 line were used (Table 6.1). The importance of aiming for monovariant-enriched parasites was because responses to heterogenous*Pf*EMP1 had the potential to mask the variant specific responses, especially when using immune sera (Vigan-Womas, Guillotte et al. 2008). Following cell sorting, the parasites were bulked up in culture for 1-2 weeks before being frozen down at both ring stage and pigmented trophozoite stage for shipment to Kilifi, Kenya. Prior to freezing, the cell-sorted trophozoites were stained for FACS assay using their respective homologous anti-NTS-DBLα antibodies to check for their final *Pf*EMP1 expression level (Figure 6.1). The proportion of infected RBCs that were positive for staining with the homologous NTS-DBL α antibody was high, ranging between 68- 80%. The inability to achieve 100% homogenous population could however be due to rapid switching away of *var* genes during the 2 weeks in culture after cell sorting.

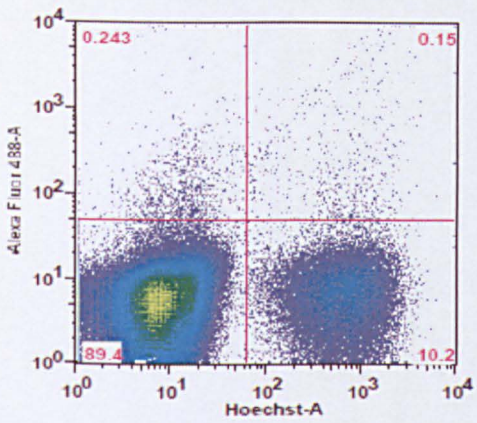
Table 6.1: *List of parasites that were cell sorted to express single variants. Also shown are the PfEMP1 variants associated with rosetting in the four laboratory strains and their IgM-binding phenotypes.*

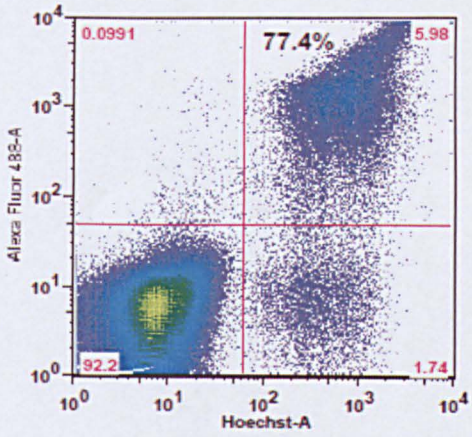
Isolate Name	*Pf*EMP1 variant	IgM binding phenotype	References
TM284R+	TM284*var*1	+	(Ghumra, Semblat et al. 2012)
HB3R+	HB3*var*6	+	(Ghumra, Semblat et al. 2012)
PAR+	IT*var*60	+	(Ghumra, Semblat et al. 2012)
R29R+	IT*var*9	-	(Ghumra, Semblat et al. 2012)
SA075	SA075*var*1	-	-

400μg Rabbit IgG

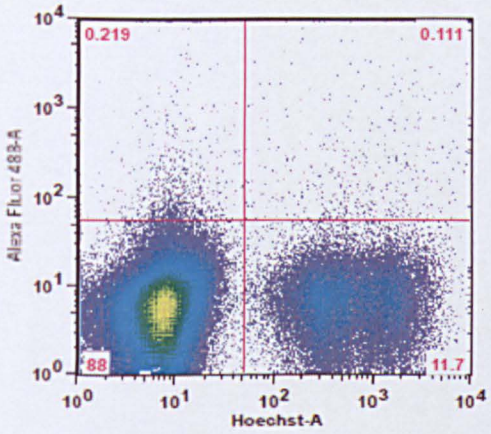
400μg NTS-DBLα

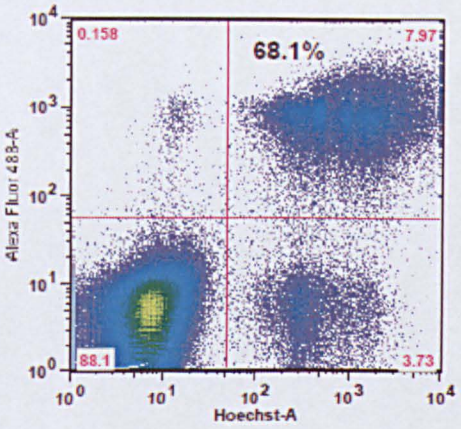
TM284R+



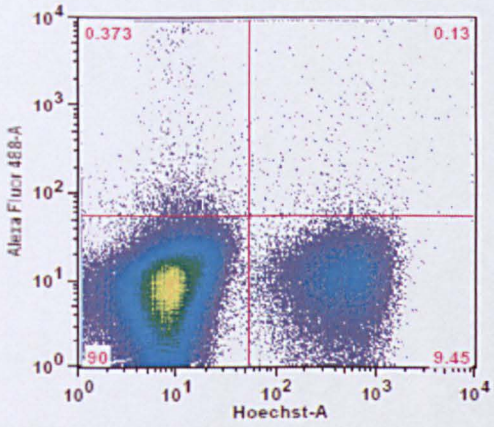


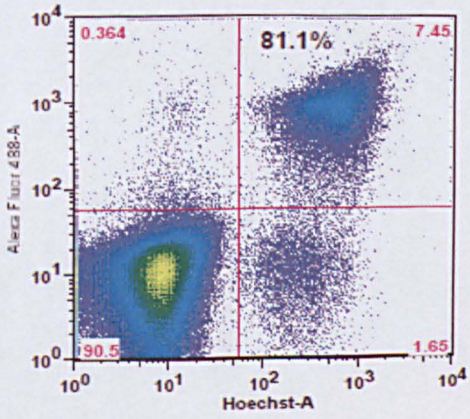
PAR+





SA075





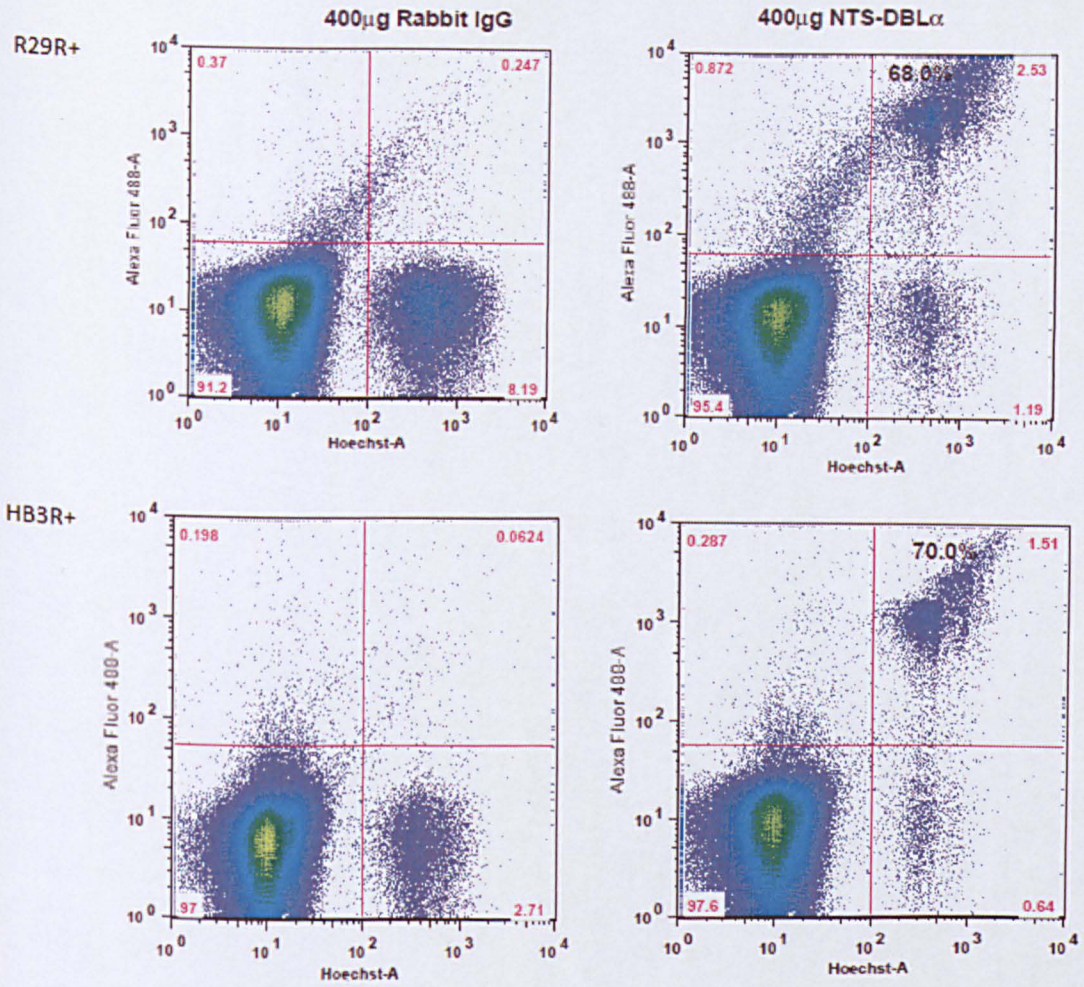


Figure 6.1: *Surface staining of rosetting parasites using homologous antibodies. The FACS assays were done 1-2 weeks after cell-sorting just before freezing down the trophozoites. On the left panel are controls stained with 400µg/ml of rabbit IgG antibody while the right panel has the parasites stained with 400µg/ml homologous NTS-DBLα antibodies. The proportion of infected RBCs that were positive for staining with the homologous NTS-DBLα antibody is shown on the top right quadrant. The FACS assay was done as previously described in Chapter 2 section 2.3.10.*

6.3.2: FACS assay using human serum

The FACS protocol described here is different from the previously described assay which used rabbit IgGs (Chapter 2, section 2.3.10). It was adapted from a protocol originally described by Bejon and colleagues in 2009 (Bejon, Warimwe et al. 2009). The aim was to examine surface recognition of *Pf*EMP1 on different cell-sorted parasites using acute and convalescent plasma pairs from children with different clinical syndromes.

To reduce the amount of day-to-day variability in the assay, the cell-sorted parasites were frozen down in several vials at trophozoite stage. On the day of the assay, a vial of the test parasite as well as the controls (listed below) were thawed out as previously described (Chapter 2, section 2.3.6). The haematocrit was then adjusted to 4% in 1X phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and ethidium bromide (10μg/ml). 11.5μl of the suspension was incubated with 1μl of each test plasma or serum in 96-well U-bottomed plates (Falcon, Becton Dickinson, USA) for 30 minutes at room temperature. This was followed by three washes using 1XPBS/0.5% BSA media and a further 30 minute incubation with 50 μl FITC-conjugated sheep anti human IgG antibody (Binding site, UK) diluted at 1:50. A final wash was done and the cells re-suspended in 200μl of wash buffer. Staining by the anti-*Pf*EMP1 rabbit antibody and the non-immune rabbit IgG were done as previously described in Chapter 2, section 2.3.10. 1000 trophozoite-infected RBCs were acquired on a FC500 flow cytometer (Beckman Coulter, UK). Data in LMD format were exported to Flowjo 7.6.1 (Tree Star Inc.) for analysis.

The following controls were run simultaneously on each plate in every experiment:-

i. Hyper immune serum (referred to as A1) against the test parasite at a dilution of 1/12.5. The serum was obtained from an adult living in Kilifi.

ii. Five non-immune European plasma against the test parasite at a dilution of 1/12.5.

iii. Hyper immune serum against A4 parasite at a dilution of 1/12.5. This is the only control that was the same in all assays regardless of the parasite being tested. It was included to check that the assay conditions remained the same when different parasites were being tested.

iv. No-antibody control stained with FITC anti-Human IgG secondary antibody.

v. 25μg/ml of rabbit anti-NTS DBL1α antibody against the homologous test parasites,

vi. 25μg/ml of rabbit IgG control antibody against the test parasites.

vii. No-antibody control stained with FITC anti-Rabbit IgG secondary antibody.

A 96-well plate layout used to perform the FACS assays is shown in Figure 6.2 where each half of the plate contained duplicate samples.

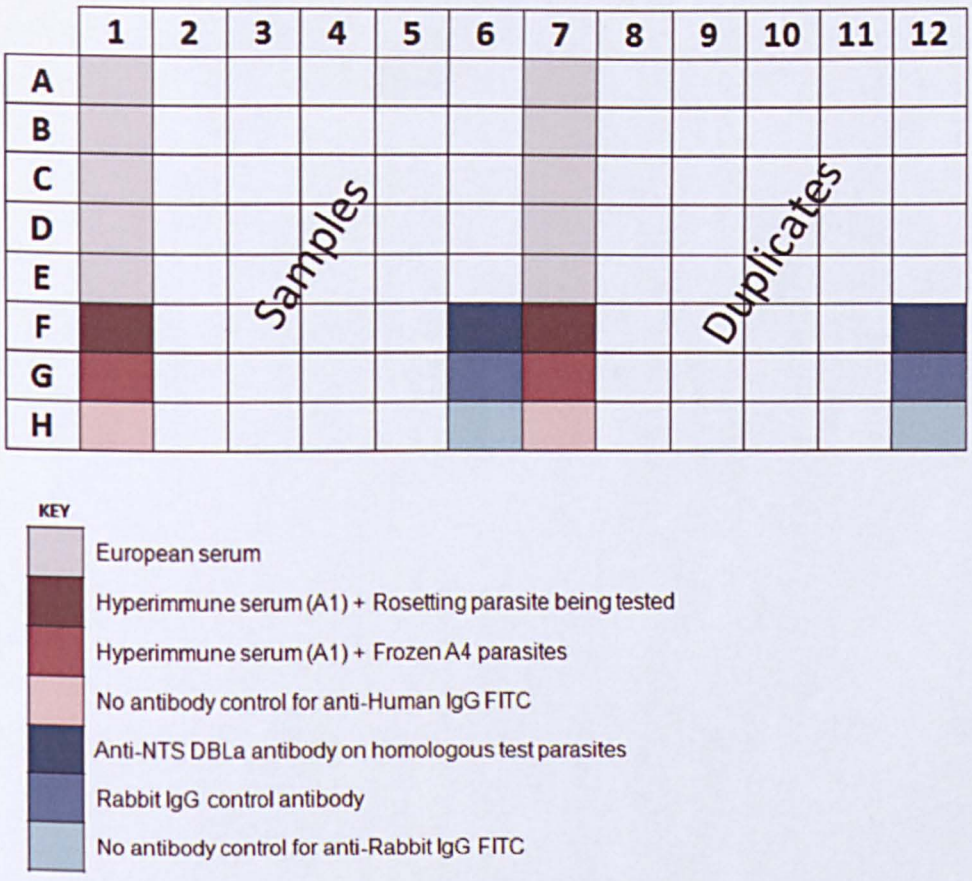


Figure 6.2: *A 96-well plate layout showing the position of samples and controls during the FACS assay. Duplicate samples and controls were arranged on either half of the plate.*

6.3.2.1 Gating strategy

Non-specific antibodies in immune plasma have previously been shown to bind various antigens expressed on the surface of RBCs (Williams and Newbold 2003). In these assays, it often resulted in a shift in the non-infected cell population as shown in Figure 6.3 A (Q4) (marked by the red dotted line). This shift in population was rarely seen in the European plasma tested on the same cells (Figure 6.3B). A gating strategy was therefore used that would correct for the shift in population.

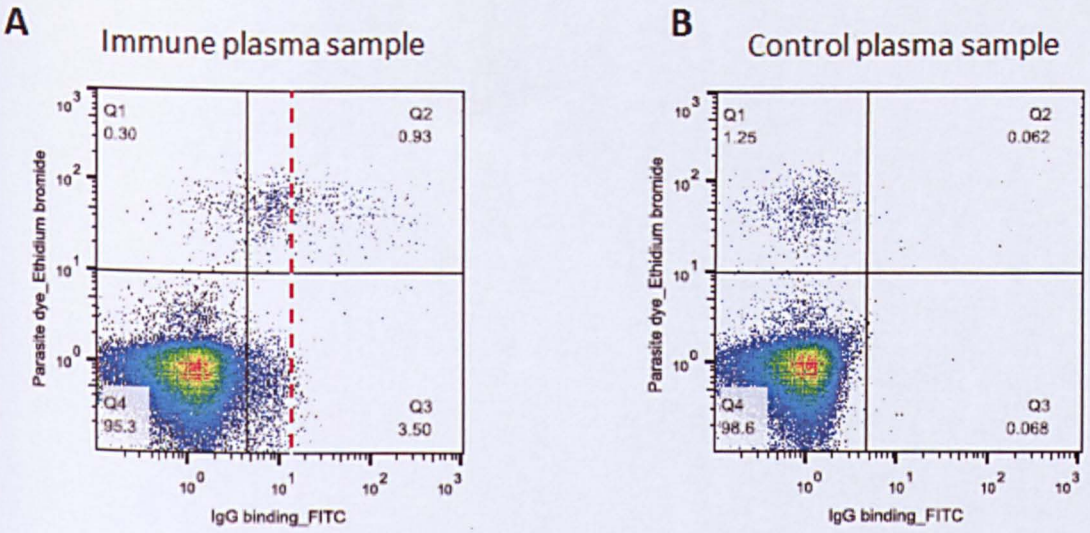
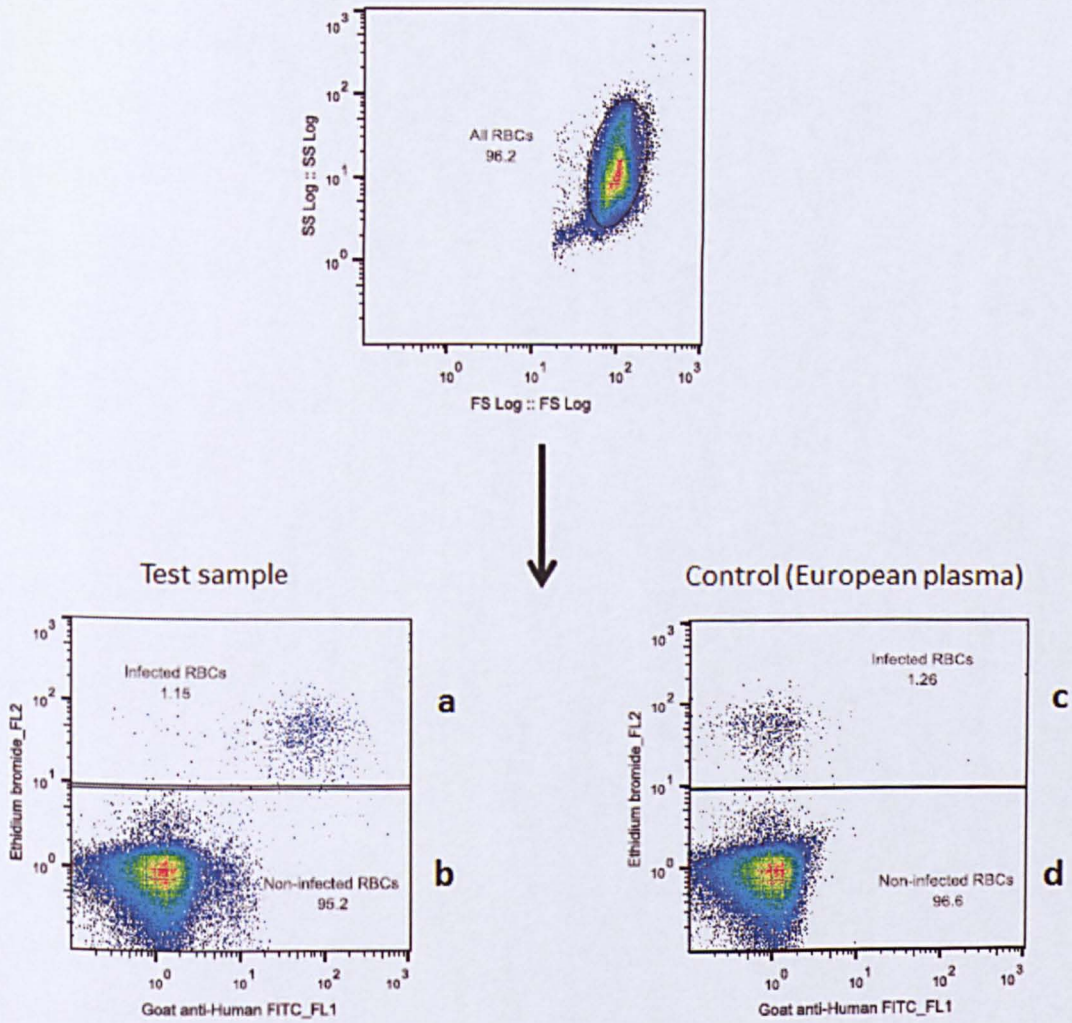


Figure 6.3: *Non-specific binding of IgG in immune plasma resulting in a shift in population of cells. Panel A shows binding by immune plasma where the red dotted line represents the shift in population of non-infected RBCs while Panel B shows binding by European plasma which does not have the shift in population. The y axis distinguishes infected RBCs from non-infected RBCs based on ethidium bromide staining while the x axis distinguishes PfEMP1 binding as FITC positive and FITC negative.*

Gating was done as follows: -

1) All RBCs were first gated based on side scatter (SSC) and forward scatter (FSC) to exclude debris or lysed cells.
2) Next, RBCs were gated by ethidium bromide_FL2 to obtain infected RBC and non-infected RBC populations
3) Finally, the median fluorescent intensity (MFI) on FL1 (Anti-Human IgG _FITC) was calculated for both infected and non-infected RBCs. To account for the non-specific shift, the MFI of non-infected RBCs was subtracted from

the MFI of infected RBCs. This was done for both the test samples and the European controls. The final variant specific IgG response was then calculated as shown in Figure 6.4.



Variant Specific antibody response= (MFI a-MFI b)-(MFI c-MFI d)

Where:-

a= Infected RBCs stained with immune plasma

b= Non-infected RBCs stained with immune plasma

c= Infected RBCs stained with non-immune plasma

d= Non-infected RBCs stained with non-immune plasma

Figure 6.4: *Calculation of variant specific antibody response. RBCs were first gated based on side scatter (SSC) and forward scatter (FSC). The MFI for infected and non-infected cells were then determined and the variant specific response calculated by subtracting the non-immune response (MFIc-MFId) from the immune response (MFI a-MFIb).*

6.3.3: Study design

Two approaches were used to investigate the antibody responses against cell-sorted parasites using plasma at acute and convalescent stages from children who had different malaria syndromes.

1. Matched case-control approach (nested study)
2. Unmatched population-based approach

6.3.3.1: Matched Case control approach

Plasma samples were selected from children who presented at the Kilifi District Hospital between 2005-2010 with varying levels of malaria severity. The clinical classifications of the different syndromes was done as previously described by Marsh and colleagues, 1995 (Marsh, Forster et al. 1995). Ten cases of pure cerebral malaria (CM) defined as impaired consciousness with a Blantyre coma score of <3 and 10 cases of pure respiratory distress (RD) defined as hemoglobin level <5 g/dL and abnormal deep breathing (English, Wale et al. 1998) were matched to uncomplicated malaria controls (UM) by age, date of admission and blood group (Table 6.2). The uncomplicated malaria controls were defined as children who were either seen in the outpatient department and required no admission or children who were admitted but did not develop any signs of severe disease throughout their admission. All patients had fever > 36.5 °C. FACS assays

were done using acute and convalescent plasma pairs for both cases and controls as described above (section 6.3.2). The acute plasma was collected from children at the time of presentation to the hospital while the convalescent samples were collected from the same children 2- 4 weeks later. Sample collection and processing were done as described in Chapter 2, section 2.2. Table 6.2 below is a list of the cases and controls with their date of admission, age, blood group and the parasite count at the time of admission. While all the cases were admitted at the hospital, the controls were either admitted or treated at the out-patient department-OPD as shown in Table 6.2.

Table 6.2: *Characteristics of samples used in the case-control study. Each severe malaria case (left column) was matched to an uncomplicated malaria control by date of admission, age, and blood group. Also shown are the parasite densities at the time of disease. The controls were either admitted in the hospital (admission) or treated at the out-patient department-OPD. All patients were assigned a unique identity (Sample ID) so that their clinical classification remained blinded until all data had been collected.*

Cerebral malaria cases

Sample ID	Date of admission	Age in months	Blood group	Parasite density/ µl
7081	24/05/2006	31	O	33856
7120	09/06/2006	86	AB	210520
7182	23/06/2006	67	A	23875
7371	10/08/2006	42	AB	81576
7864	28/02/2007	43	A	966520
7940	19/04/2007	43	A	13502
8211	05/08/2007	31	O	679320
8710	07/07/2008	19	O	118440
8948	13/10/2008	27	O	29900
7250	08/07/2006	46	AB	294060

Controls

Sample ID	Date of admission	Age in months	Blood group	Parasite density/ µl	Admission/OPD
7044	03/05/2006	31	O	817920	OPD
7324	26/07/2006	80	A	9471	Admission
7057	16/05/2006	68	A	1231580	Admission
7211	29/06/2006	40	A	397440	Admission
7726	12/03/2007	44	B	473020	Admission
8210	09/04/2007	50	A	132840	Admission
8208	04/08/2007	34	O	109980	Admission
8624	11/06/2008	20	O	18088	OPD
8344	12/09/2008	26	O	100100	OPD
7384	15/08/2006	39	A	18000	OPD

Respiratory distress cases

Sample ID	Date of admission	Age in months	Blood group	Parasite density/ µl
7214	29/06/2006	24	A	248400
7799	31/02/2007	42	O	369720
8140	29/07/2007	12	B	86460
7116	07/06/2006	37	A	155100
7157	17/06/2006	40	A	803400
9215	06/04/2009	18	A	599040
9423	03/06/2009	31	AB	257740
9978	28/12/2009	88	B	86800
10012	03/01/2010	65	A	50260
7242	06/07/2006	25	A	358560

Controls

Sample ID	Date of admission	Age in months	Blood group	Parasite density/ µl	Admission/OPD
7069	19/05/2006	24	B	927080	Admission
8120	20/07/2007	51	O	222040	Admission
7792	29/01/2007	16	A	26432	Admission
7115	07/06/2006	32	AB	139080	Admission
7067	19/05/2006	34	AB	1020600	Admission
9197	29/03/2009	27	AB	214200	OPD
9254	20/04/2009	44	A	20700	OPD
9661	17/08/2009	41	A	2025300	OPD
9437	08/06/2009	42	A	237360	OPD
7377	14/08/2006	26	B	1487640	Admission

6.3.3.2: Unmatched-Population-based approach

Like in the case-control approach, plasma samples were selected from children who attended Kilifi District Hospital with varying levels of severity and clinical syndromes. To differentiate it from the case-control study, this study was referred to as "population-based approach". It included a bigger sample size with the acute-convalescent pairs of plasma being obtained at two time periods: 1994-1996 (53 plasma pairs) and 2005-2010 (163 plasma pairs). However, unlike the case-control study, this approach included children with overlapping clinical syndromes (See tables 6.3 and 6.4) and analysis involved comparing all severe cases against the non-severe cases.

There were important differences between the two sampling periods. First, a steady decline in malaria transmission that has been observed in Kilifi the last 20 years (Okiro, Hay et al. 2007; O'Meara, Bejon et al. 2008), and which might have affected host-pathogen interaction patterns between the two time-points. Secondly, the prevalence of clinical syndromes during the two sampling points (Table 6.3 and 6.4) was different: 1994-1996 samples had more SMA cases (18.9% versus 3.7%) while the 2005-2010 samples had more RD cases (9.8% versus1.8 %). Thirdly, whether or not this is due to changes in transmission patterns, there seems to be an overall increase the average age in children who present to the hospital with different clinical syndromes (mean age between 1994-1996 was 37.07±32.45 months while the mean age between 2005-2010 was 48.302±43.14 months).

Patients were assigned unique identification numbers and their clinical classification remained blinded until all data had been collected. Some of the plasma samples, especially at the acute phase, have been used in previous studies by Warimwe *et al.*, 2009 and 2012 (Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012).

Table 6.3: Clinical characteristics and frequency of samples collected between 1994-1996

Syndrome	Mean Age (months)_±SD	Frequency (N)
Respiratory distress (RD)	31.0	1.8% (1)
Impaired consciousness (IC) (9 of whom were CM cases)	36.16± 22.1	22.6% (12)
Severe malarial anaemia (SMA)	33.91±13.7	18.9%(10)
Respiratory distress/ Impaired consciousness/ Severe malarial anemia (RD/IC/SMA)	24.0	1.8%(1)
Non-severe malaria (NS)	38.83±14.9	54.7%(29)
TOTAL		**100% (53)**

Table 6.4: Clinical characteristics and frequency of samples collected between 2005-2010

Syndrome	Mean Age (months)_±SD	Frequency (N)
Respiratory distress (RD)	42.17±15.6	9.8 (16)
Impaired consciousness (IC) (17 of whom were CM cases)	47.31±32.7	18.4 % (30)
Severe malarial anemia (SMA)	26.58±14.5	3.7% (6)
Respiratory distress/ Impaired consciousness (RD/IC)	37.02±18.4	7.4% (12)
Respiratory distress/ Impaired consciousness/ Severe malarial anemia (RD/IC/SMA)	30.29	1.2% (2)
Severe malarial anemia/ Respiratory distress (SMA/RD)	42.06±25.0	1.2% (2)
Impaired consciousness/ Severe malarial anemia (IC/SMA)	57.7	0.6% (1)
Non-severe malaria (NS)	51.09±31.5	57.7% (94)
TOTAL		**100% (163)**

6.3.4: Data analysis

Data analysis was done using both Stata™ version 11.0 software and Graphpad prism 6, where P value <0.05 was considered significant for all tests. In stata, raw data were imported into the software and a "do-file" was written that was used for subsequent analyses. Spearman's rank correlation was used to test associations between two continuous variables while the relationships between a continuous

and a categorical variable were tested by either Mann-Whitney U test or Kruskal-Wallis test.

6.4: RESULTS

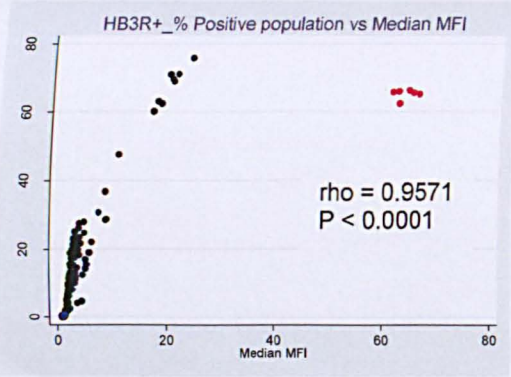
6.4.1: Comparison of mean fluorescent intensity, median fluorescent intensity and % positive population by FACS analysis

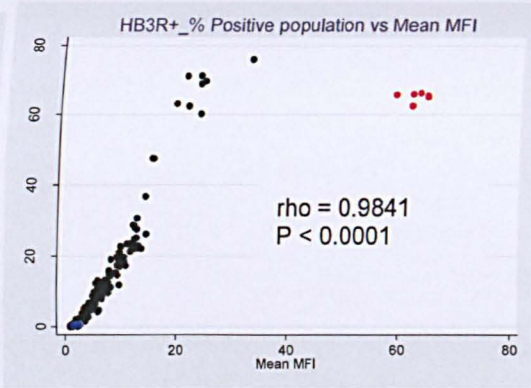
Three different FACS generated measures were used to quantify the variant specific IgG responses in the plasma. These were:-mean fluorescent intensity, median fluorescent intensity and percentage positive population. The three measures were compared against each other in turn to determine their level of correlation in each of the parasite strains that was tested. The correlation was done on all children's samples in the case-control study (Figure 6.5 in black) as well as rabbit non-immune IgG (in blue) and variant-specific IgG (in red). Figure 6.5 shows scatter plots for results involving HB3R+, PAR+, R29+, SA075 and TM284R+. Spearman's rank correlation coefficient (Rho) and the P values are shown on each graph.

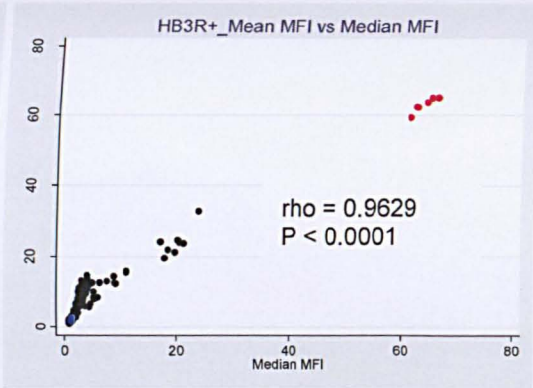
There was a significant positive correlation for all the three measures against each other in all the assays (range of Rho- 0.4189-0.9841). HB3R+ and PAR+ showed the highest level of correlation with Rho values>0.9. However the variant-specific rabbit IgG response against HB3R+ formed a population of cells off the linear fit (Figure 6.5, Panel 1) when % percentage positive population was correlated with both mean and median. A positive correlation was seen for all isolates even when the controls were excluded from the analysis. (Range of Rho was 0.3234-0.9778).

Although a positive correlation would suggest that any of the three measures could be used in analysis, median fluorescence intensity henceforth referred to as "MFI", was chosen for subsequent analysis due to the wide range in some of the reads.

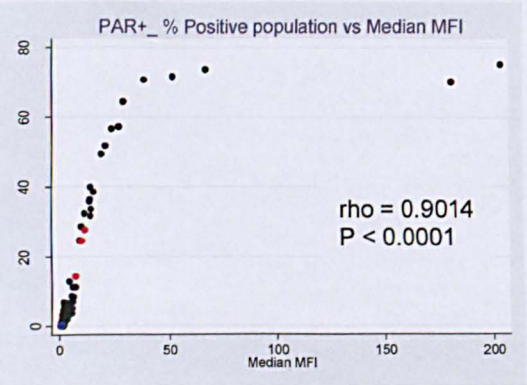
1. ***HB3R+***

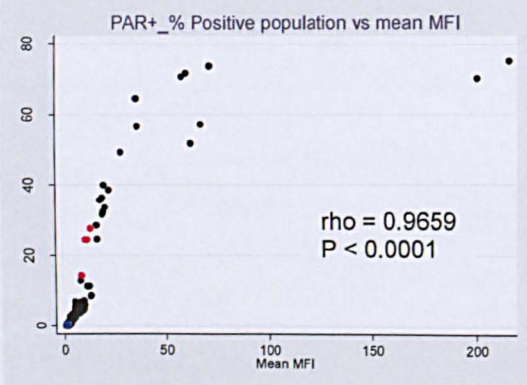

HB3R+_% Positive population vs Median MFI
rho = 0.9571
P < 0.0001
Median MFI

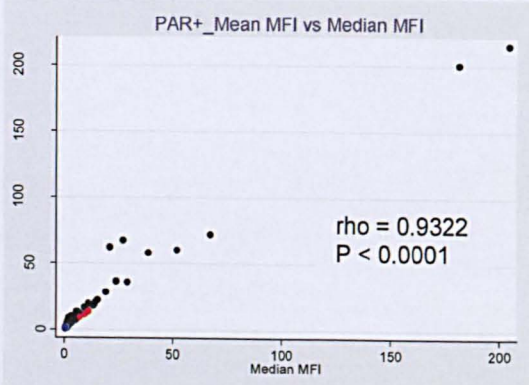

HB3R+_% Positive population vs Mean MFI
rho = 0.9841
P < 0.0001
Mean MFI


HB3R+_Mean MFI vs Median MFI
rho = 0.9629
P < 0.0001
Median MFI

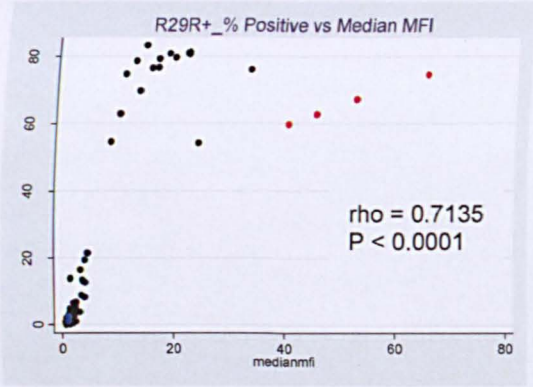
2. PAR+

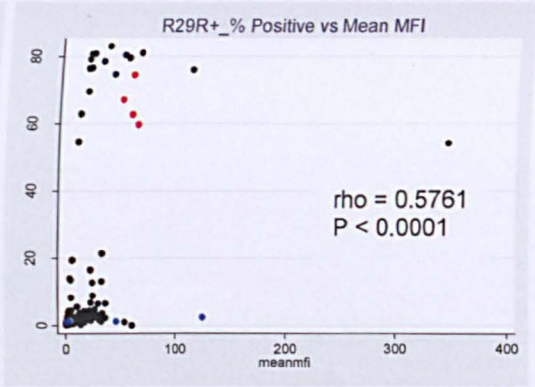

PAR+_ % Positive population vs Median MFI
rho = 0.9014
P < 0.0001
Median MFI

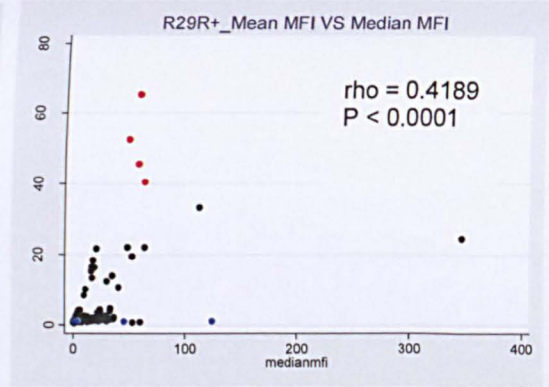

PAR+_% Positive population vs mean MFI
rho = 0.9659
P < 0.0001
Mean MFI


PAR+_Mean MFI vs Median MFI
rho = 0.9322
P < 0.0001
Median MFI

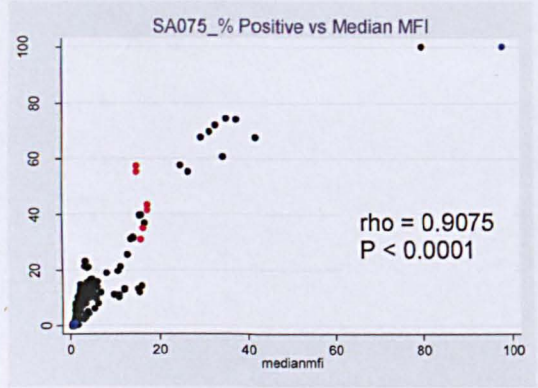
3. *R29R+*

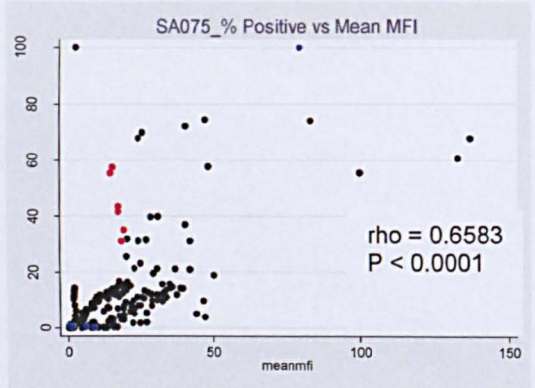


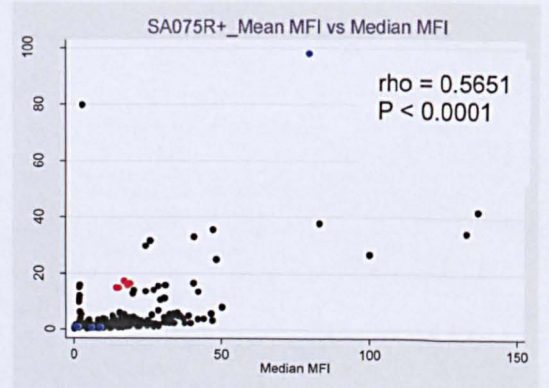




4. SA075







5. TM284R+

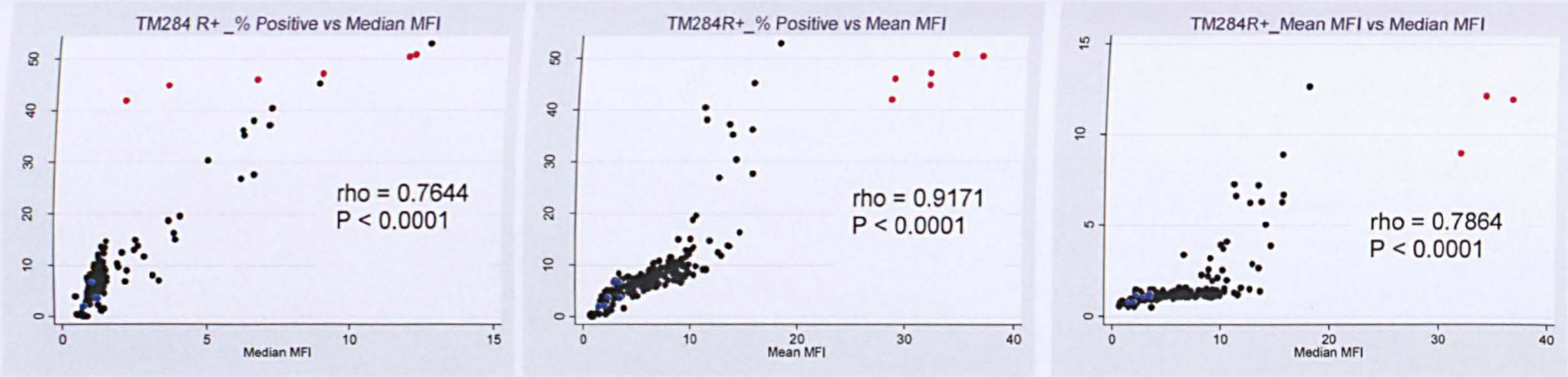


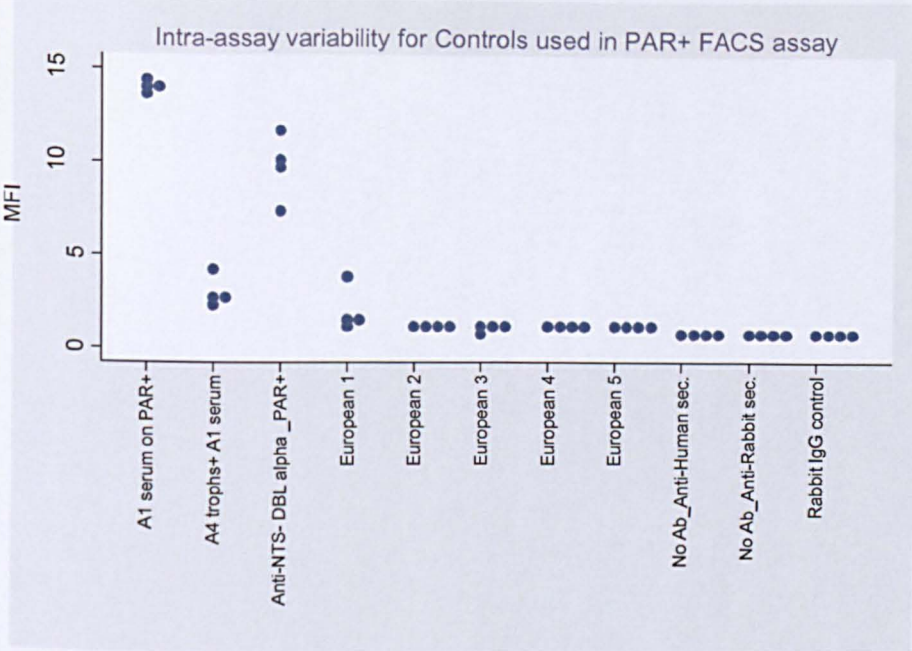
Figure 6.5: *Correlation between mean fluorescence intensity (Mean MFI), median fluorescence intensity (Median MFI) and percent positive population for FACS assays carried out on different parasites. For each of the assays, correlations were done for 1) % positive population versus Median MFI, 2) % positive population versus mean MFI and 3) mean MFI versus median MFI. Shown on the graphs are the Spearman's rank correlation coefficient (Rho) and the P value. Variant specific Anti-PfEMP1 rabbit antibodies are represented in red while the control non-immune rabbit IgG are represented in blue. The rest of the other samples are represented in black.*

6.4.2: Determination of inter-assay and intra-assay variability

Due to the large number of samples and controls involved, assays were carried out on different days. Assay variability was therefore determined for all the isolates. Coefficient of variation (% CV) which is a measure of the dispersion of data points around the mean, was calculated for duplicate reads within each plate for all the 11 controls. Although there are no generally accepted minimal performance requirements for immunoassays, the industrial standard for % CV that has been used for ELISA is <20% (USDHHS 2001). In the current assay, an arbitrary %CV cut-off was set at 20 and any value less than this considered acceptable. Figure 6.6 shows the MFI for all the 11 controls in the different assays. Each point on the graph represents a read from an individual well. The table below each graph shows the average MFI for the duplicate reads in each plate and their % CV.

Results showed that 92% of all the % CV values were within the acceptable value of less than 20% with the negative controls generally showing lesser variability than the positive controls. In most cases, the outlier reads occurred in one of the duplicate wells in a plate. However, data from all sample wells were included in the analysis.

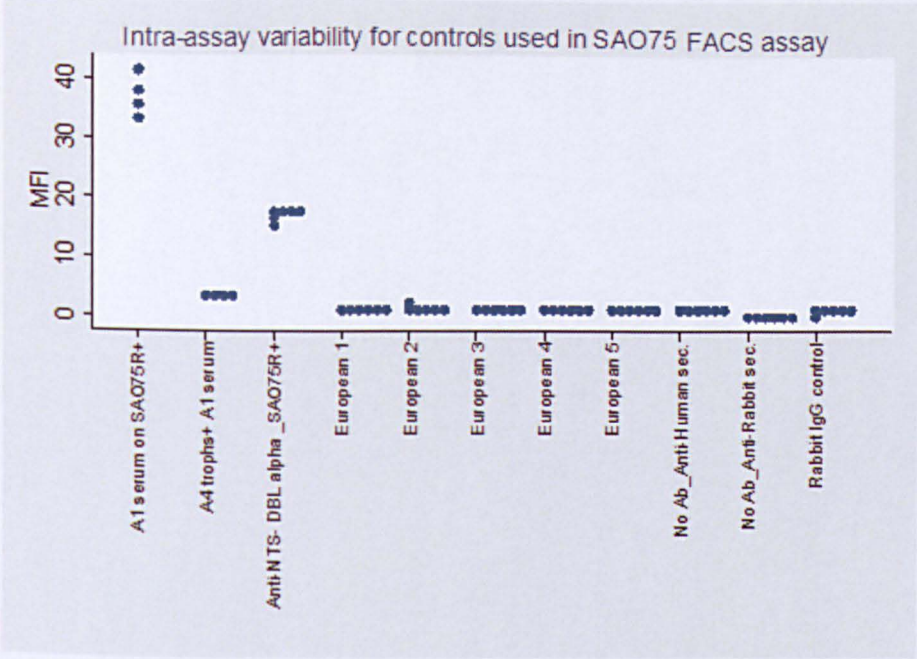
1. PAR+



% CV FOR DUPLICATE READS IN FACS ANALYSIS _PAR+ ACROSS 2 PLATES

	Plate 1		Plate 2	
Control	**Mean**	**% CV**	**Mean**	**% CV**
A1 serum on PAR+	14.200	0.996	14.000	0.000
A4 trophs+ A1 serum	2.485	16.788	3.580	30.812
European 1	1.335	15.360	2.775	58.862
European 2	1.135	3.115	1.285	3.852
European 3	1.064	16.747	1.195	5.325
European 4	1.160	18.287	1.150	12.298
European 5	1.135	1.869	1.200	1.179
Anti-NTS- DBL alpha _PAR+	10.900	9.082	8.530	18.403
No Ab_Anti-Human secondary Ab.	0.928	4.955	0.917	2.313
No Ab_Anti-Rabbit secondary Ab.	0.688	0.309	0.747	2.083
Rabbit IgG control	0.839	2.446	0.897	3.153

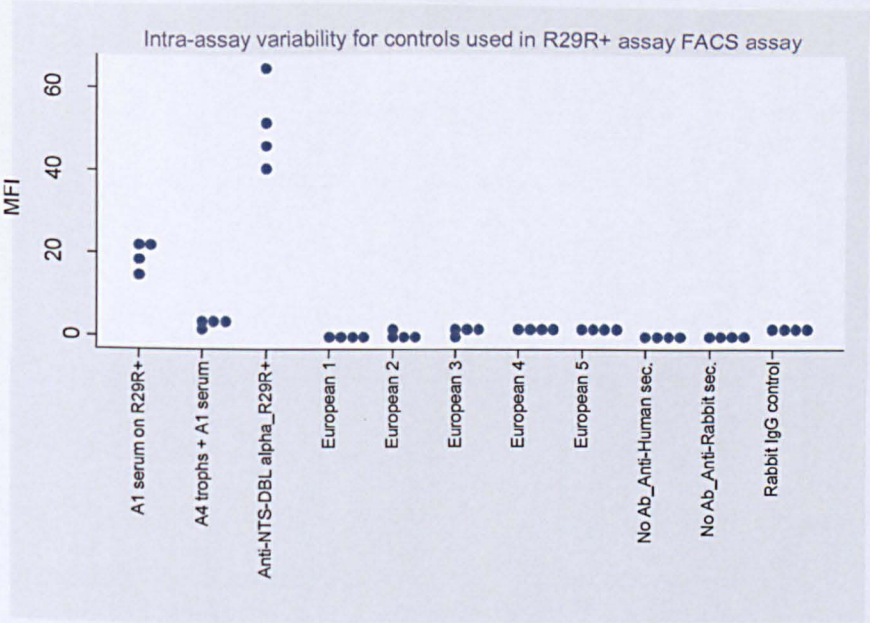
2. SA075



% CV FOR DUPLICATE READS IN FACS ANALYSIS _SAO75 ACROSS 3 PLATES

	Plate 1		Plate 2		Plate 3	
Control	**Mean**	**% CV**	**Mean**	**% CV**	**Mean**	**% CV**
A1 serum on SAO75	39.900	7.443	34.200	4.962	ND	ND
A4 trophs+ A1 serum	3.615	9.585	3.310	7.263	ND	ND
European 1	0.834	6.363	0.816	13.345	0.959	26.692
European 2	0.647	5.359	0.865	9.406	1.849	78.856
European 3	0.737	3.168	0.947	7.994	0.775	5.569
European 4	0.800	10.253	1.035	0.683	0.854	0.331
European 5	0.789	6.188	0.917	3.009	0.785	7.927
Anti-NTS- DBL alpha _SA075	16.150	2.189	17.400	0.000	17.600	0.804
No Ab_Anti-Human secondary Ab.	0.637	5.772	0.738	6.132	0.648	6.225
No Ab_Anti-Rabbit secondary Ab.	0.379	10.275	0.576	1.597	0.493	7.609
Rabbit IgG control	0.584	5.938	0.769	2.023	0.134	9.071

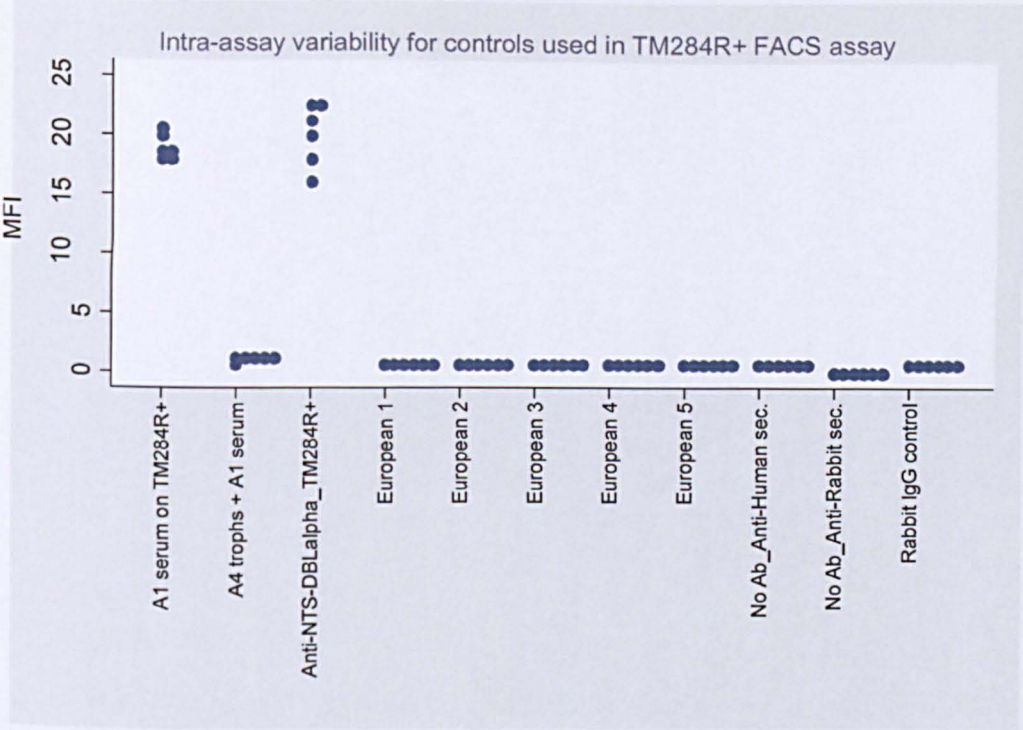
3. R29+



% CV FOR DUPLICATE READS IN FACS ANALYSIS _R29R+ ACROSS 2 PLATES

	Plate 1		Plate 2	
Control	**Mean**	**% CV**	**Mean**	**% CV**
A1 serum on R29R+	20.600	9.441	18.350	4.455
A4 trophs + A1 serum	2.755	1.063	3.975	0.290
Anti-NTS-DBL alpha_R29R+	43.050	19.831	59.050	8.980
European 1	0.791	0.377	0.823	0.040
European 2	0.883	0.410	0.867	0.070
European 3	0.934	0.462	0.902	0.085
European 4	0.972	0.482	0.994	0.052
European 5	0.967	0.464	0.970	0.022
No Ab_Anti-Human secondary Ab.	0.733	0.287	0.857	0.009
No Ab_Anti-Rabbit secondary Ab.	0.681	0.338	0.644	0.030
Rabbit IgG control	1.075	0.514	1.025	0.007

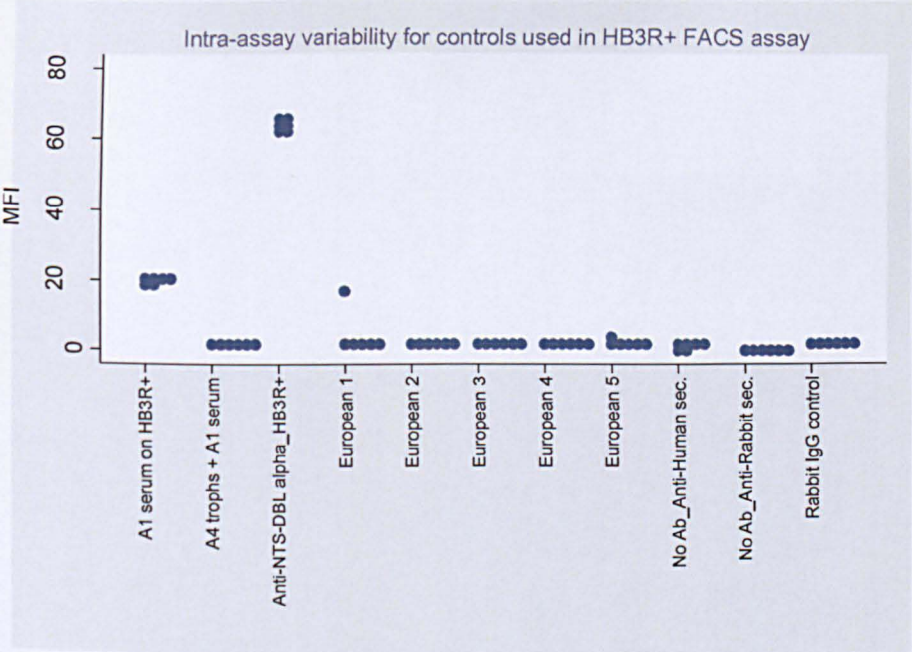
4. TM284R+



% CV FOR DUPLICATE READS IN FACS ANALYSIS _TM284R+ ACROSS 3 PLATES

	Plate 1		Plate 2		Plate 3	
Control	**Mean**	**% CV**	**Mean**	**% CV**	**Mean**	**% CV**
A1 serum on TM284R+	20.250	0.349	18.100	2.344	18.200	2.331
A4 trophs + A1 serum	1.080	5.238	1.095	3.229	0.949	6.486
Anti-NTS-DBLalpha_TM284R+	18.700	7.563	19.250	23.876	0.655	6.590
European 1	0.679	2.397	0.702	0.101	22.050	4.169
European 2	0.693	5.616	0.684	5.582	0.685	2.169
European 3	0.641	3.751	0.634	2.121	0.671	3.269
European 4	0.752	3.670	0.695	5.291	0.607	7.921
European 5	0.749	3.965	0.710	3.785	0.649	9.486
No Ab_Anti-Human secondary Ab.	0.640	0.110	0.539	42.413	0.573	7.781
No Ab_Anti-Rabbit secondary Ab.	0.273	23.613	0.294	9.621	0.230	20.643
Rabbit IgG control	0.612	1.041	0.721	8.538	0.576	1.964

5. HB3R+



% CV FOR DUPLICATE READS IN FACS ANALYSIS _HB3R+ ACROSS 3 PLATES

	Plate 1		Plate 2		Plate3	
Control	**Mean**	**% CV**	**Mean**	**% CV**	**Mean**	**% CV**
A1 serum on HB3R+	20.450	1.037	20.550	5.161	18.200	2.331
A4 trophs + A1 serum	1.520	3.722	1.670	6.775	1.315	1.613
Anti-NTS-DBL alpha_HB3R+	64.900	0.872	62.250	1.477	64.500	4.166
European 1	9.220	120.868	1.460	10.655	1.295	0.546
European 2	1.295	2.730	1.290	0.000	1.290	4.385
European 3	1.290	0.000	1.250	1.131	1.250	3.394
European 4	1.430	1.978	1.385	0.511	1.480	4.778
European 5	1.365	1.554	1.485	2.381	2.535	66.666
No Ab_Anti-Human secondary Ab.	1.025	2.070	1.025	0.690	0.914	0.464
No Ab_Anti-Rabbit secondary Ab.	0.890	2.146	0.886	1.038	0.807	2.629
Rabbit IgG control	1.245	2.840	1.220	2.318	1.110	1.274

Figure 6.6: *Intra-assay variability for the 11 controls used in the case-control FACs assays for PAR+, SA075, R29R+, TM284R+ and HB3R+. Each point in the graph represents an individual read. The table below each graph shows the mean and % CV for the duplicate reads in each plate.*

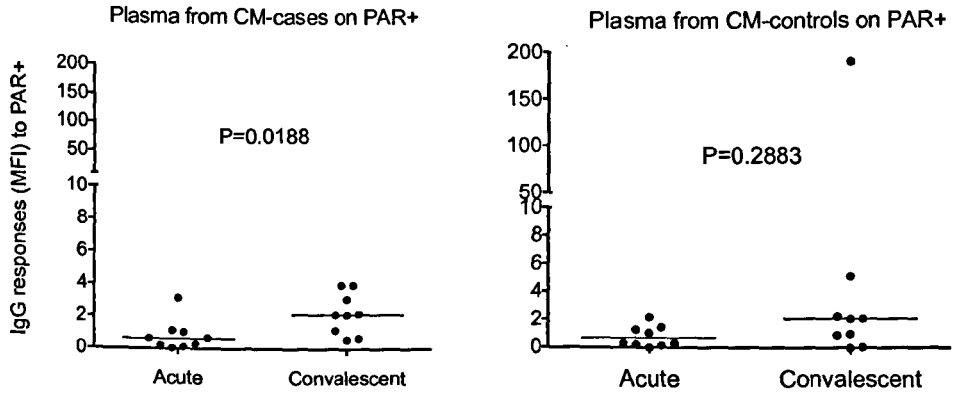
6.4.3: Surface recognition of parasites selected to express single variants by IgGs from children with different malaria syndromes-case control study

Variant-specific antibody responses to rosette-selected parasites were examined. Acute and convalescent plasma pairs from children who presented with pure CM and pure RD were compared against their matched UM controls. Figures 6.7 and 6.8 shows the median of MFI values for antibody responses of the cases and controls at both acute and convalescent stages in CM and RD cases respectively. Each data point represents plasma from one patient. P value <0.05 was considered statistically significant by Mann-Whitney U-test. A clinically important variant would be expected to show an increase in convalescent antibody responses in cases but not in the controls.

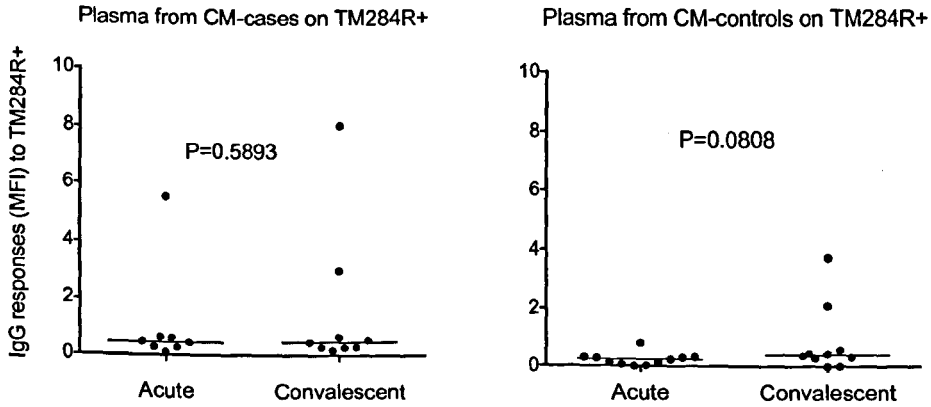
Results showed a significant increase in antibody responses to the PAR+ parasite at acute versus convalescent in the CM cases (Figure 6.7 Panel A, P=0.0188) but not in the controls (P=0.2883). A similar significant increase was seen against HB3R+ parasites in RD cases (Figure 6.8 Panel C, P=0.0089) but not in the controls (P=0.0648). These results suggest that the two variants, IT*var*60 expressed by PAR+ and HB3*var*6 expressed by HB3 could be clinically important. It could be that children who presented with pure CM and RD syndromes were infected with parasites that were antigenically related to PAR+ and HB3R+ respectively.

Interestingly, results from some of the UM cases also showed variant specific responses. Such results where convalescent responses were significantly higher than the acute responses were seen for RD controls tested against R29R+ (P=0.0354) and TM284R+ (P=0.0291) parasites.

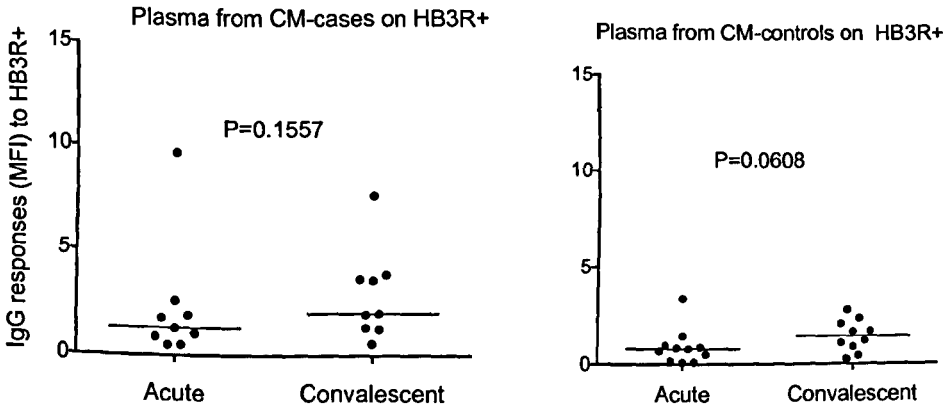
A


Plasma from CM-cases on PAR+
IgG responses (MFI) to PAR+
200
150
100
50
10
8
6
4
2
0
P=0.0188
Acute
Convalescent
Plasma from CM-controls on PAR+
200
150
100
50
10
8
6
4
2
0
P=0.2883
Acute
Convalescent

B


Plasma from CM-cases on TM284R+
IgG responses (MFI) to TM284R+
10
8
6
4
2
0
P=0.5893
Acute
Convalescent
Plasma from CM-controls on TM284R+
10
8
6
4
2
0
P=0.0808
Acute
Convalescent

C


Plasma from CM-cases on HB3R+
IgG responses (MFI) to HB3R+
15
10
5
0
P=0.1557
Acute
Convalescent
Plasma from CM-controls on HB3R+
15
10
5
0
P=0.0608
Acute
Convalescent

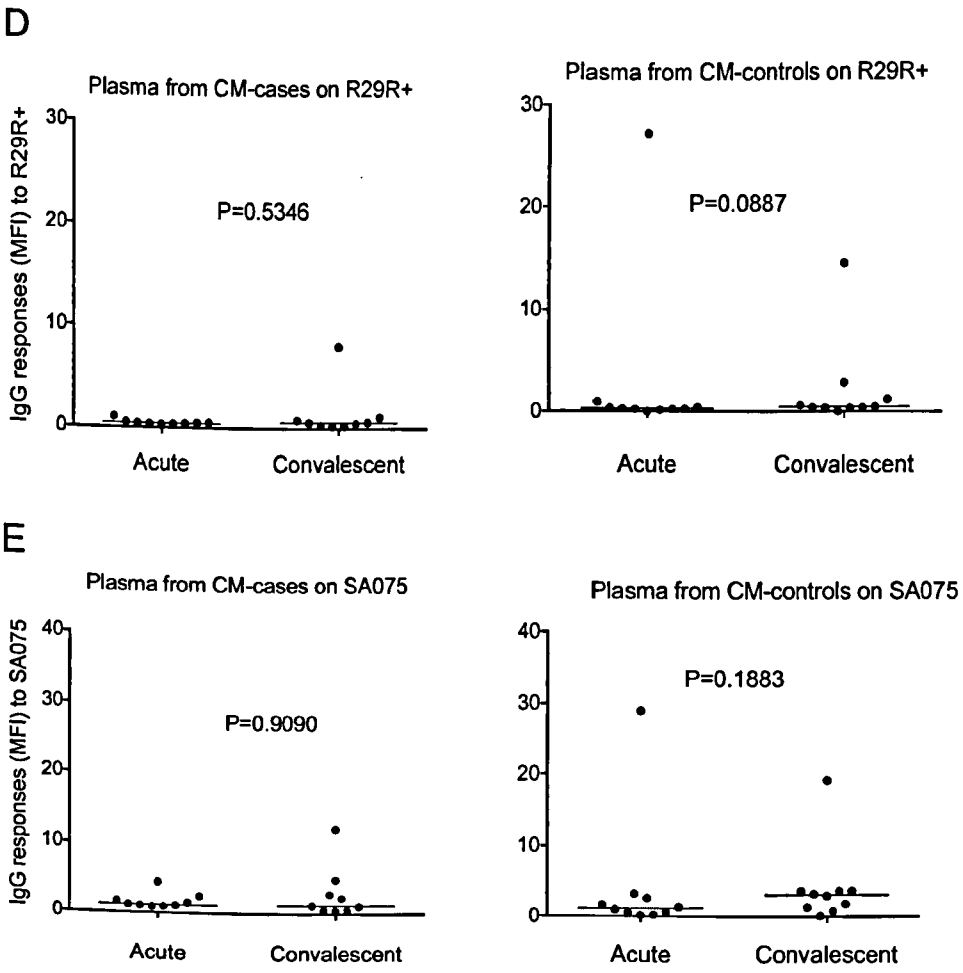
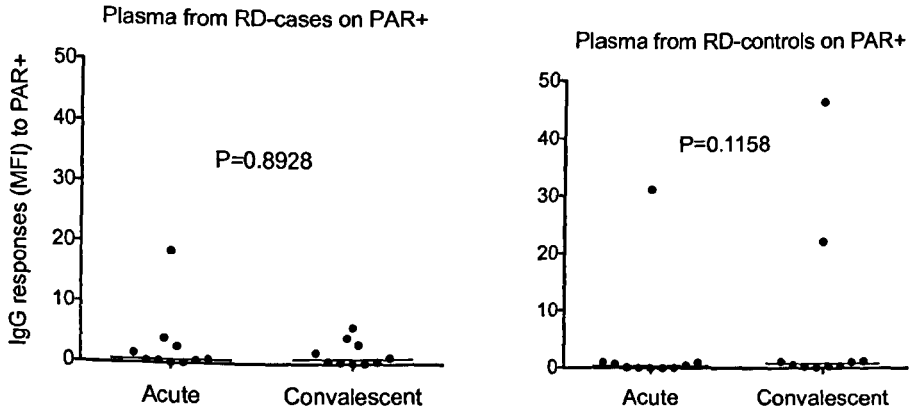
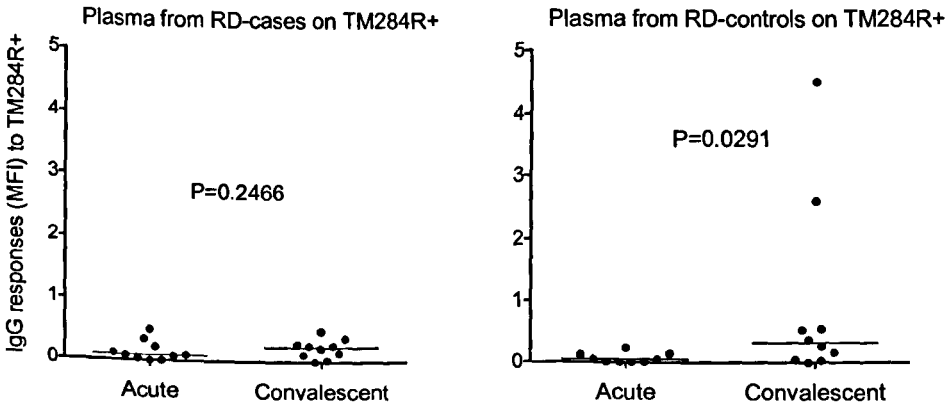


Figure 6.7: *Surface recognition of cell sorted parasite lines by plasma from children with pure cerebral malaria-CM (left panel) and their uncomplicated malaria -UM controls (right panel) at both acute and convalescent stages. Shown on the graph are the median of the MFIs with each data point representing plasma from one patient. The parasites used were A) PAR+ B) TM284R+C) HB3R+ D) R29R+ and E) SA075. (Mann Whitney U test, P<0.05). Only PAR+ showed a significant difference in acute versus convalescent responses in the cases and not in the controls.*

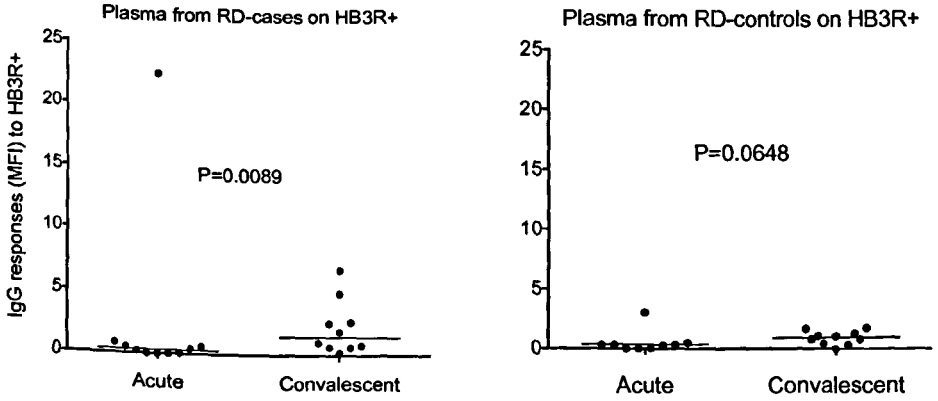
A


Plasma from RD-cases on PAR+
IgG responses (MFI) to PAR+
50
40
30
20
10
0
P=0.8928
Acute
Convalescent
Plasma from RD-controls on PAR+
50
40
30
20
10
0
P=0.1158
Acute
Convalescent

B


Plasma from RD-cases on TM284R+
IgG responses (MFI) to TM284R+
5
4
3
2
1
0
P=0.2466
Acute
Convalescent
Plasma from RD-controls on TM284R+
5
4
3
2
1
0
P=0.0291
Acute
Convalescent

C


Plasma from RD-cases on HB3R+
IgG responses (MFI) to HB3R+
25
20
15
10
5
0
P=0.0089
Acute
Convalescent
Plasma from RD-controls on HB3R+
25
20
15
10
5
0
P=0.0648
Acute
Convalescent

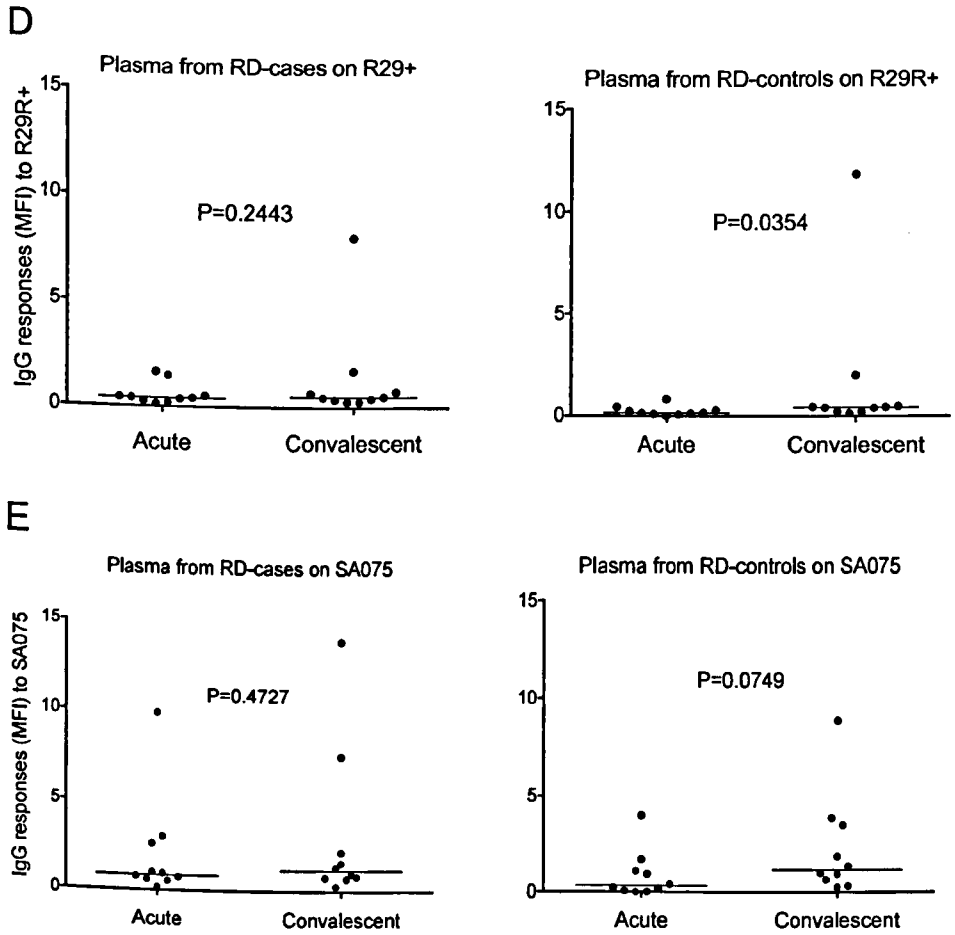


Figure 6.8: *Surface recognition of cell sorted parasite lines by IgGs from children with pure respiratory distress-RD (left panel) and their uncomplicated malaria-UM controls (right panel) at both acute and convalescent stages. Shown on the graph are the median of the MFIs with each data point representing plasma from one patient. The parasites used were A) PAR+ B) TM284R+C) HB3R+ D) R29R+ and E) SA075. (Mann Whitney U test, P<0.05). Only HB3R+ showed significant difference in acute versus convalescent responses in the cases and not in the controls.*

6.4.4: Surface recognition of parasites enriched in a single variant by IgGs from children with different malaria syndromes-Population-based study

Due to the limited number of matched samples in the case control study, analysis of antibody responses was further extended to include more samples obtained from the hospital. Samples were obtained at two different sampling time points: - 1994-1996 and 2005-2010. Analysis included all impaired consciousness (IC) cases, as opposed to those with pure CM only. Also included were patients who presented with overlapping syndromes. Each acute sample had its convalescent pair that was collected 2-4 weeks later. Plasma samples collected between 1994-1996 (N=53 pairs) were tested against 4 parasite lines i.e. PAR+, HB3R+, R29R+ and SA075 while samples collected between 2005-2010 (N=163 pairs) were tested against two parasite lines i.e. SA075 and PAR+. Due to the large number of samples involved, assays for each parasite were tested in 9 plates which were analyzed over 2 days. Variability was therefore based on both inter-assay and intra-assay. (Assay variability data in appendix 8.6).

Analysis was first done by pooling all severe malaria cases including those with pure and overlapping syndromes into one group "severe cases" and comparing their antibody responses (separate acute and convalescent) against the non-severe malaria group. In the 1994-1996 dataset, only in R29R+ was there a significant difference in IgG responses between the acute and convalescent in the severe cases (P=0.0404) (Figure 6.9). In the 2005-2010 data, the convalescent antibody responses against SA075 were significantly higher than the acute responses in both the severe and non-severe cases (P=0.0157 and P=0.0032 respectively) (Figure 6.10). On the other hand, convalescent responses against PAR+ were significantly higher than the acute responses only in the severe cases but not in the non-severe cases (P=0.0131).

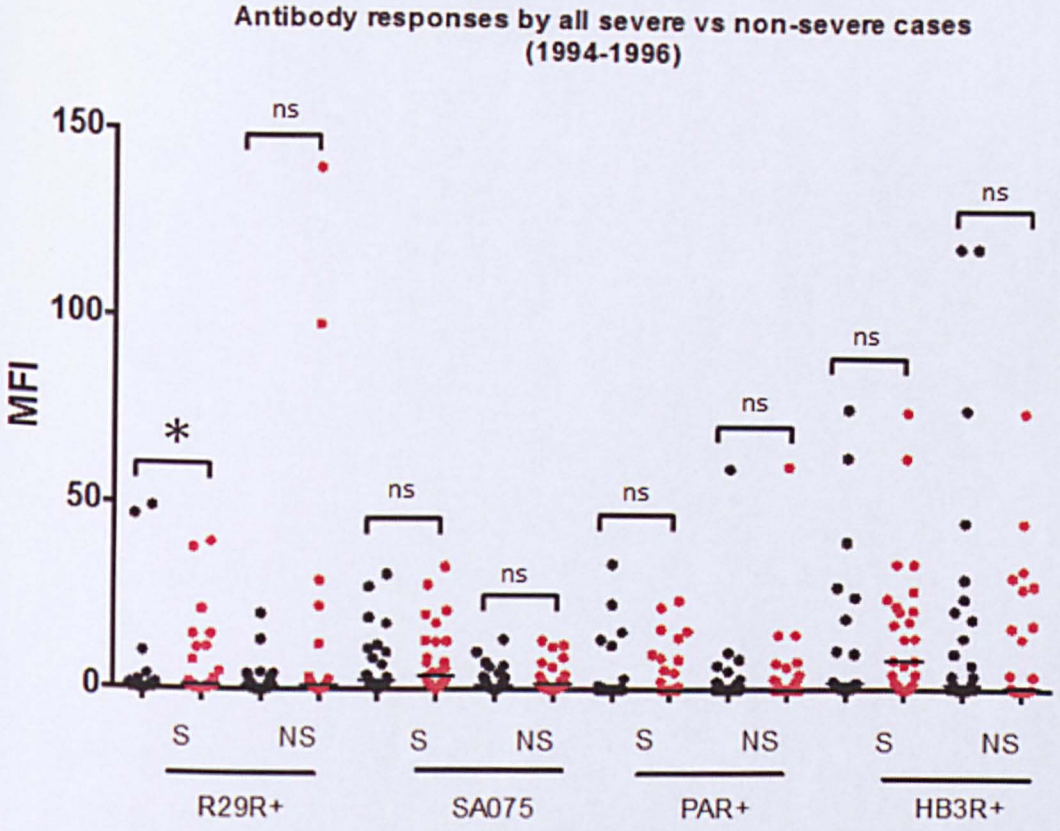


Figure 6.9: *Surface recognition of four selected P.falciparum parasite strains:- R29R+, SA075, PAR+ and HB3R+ and by acute (black) and convalescent (red) plasma from severe (S) and non-severe(NS) cases in 1994-1996.Only R29R+ showed a difference between acute and convalescence in severe cases. Statistically significant p values were represented with an asterix where *p value<0.05. (ns) represents values that were not statistically different.*

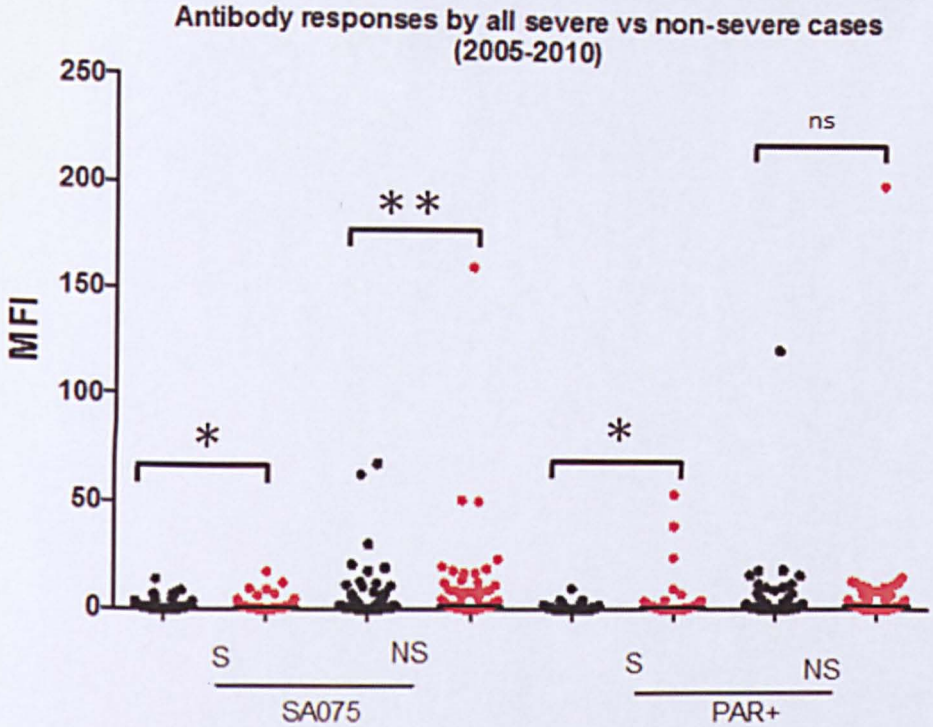
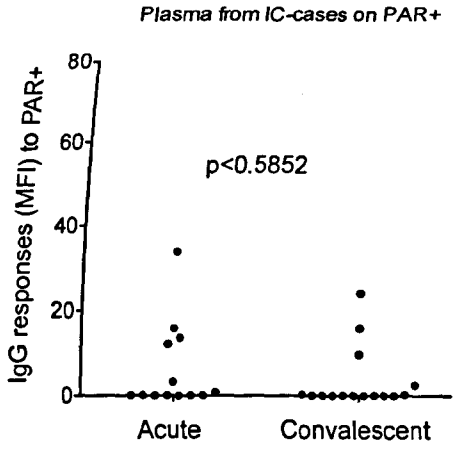


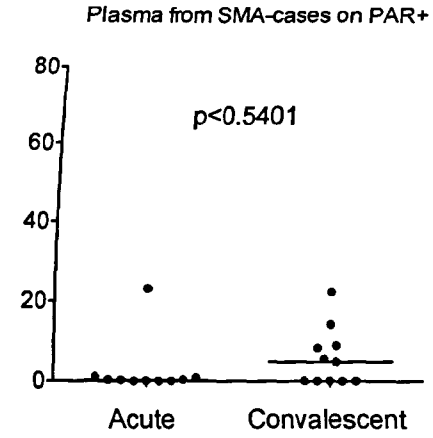
Figure 6.10: *Surface recognition of two rosette selected parasites:- SA075 and PAR+ by acute (black) and convalescent (red) plasma from severe (S) and non-severe(NS) cases in 2005-2010. Statistically significant p values were represented with an asterix where *p value<0.05 and **p value<0.005. (ns) represents values that were not statistically different.*

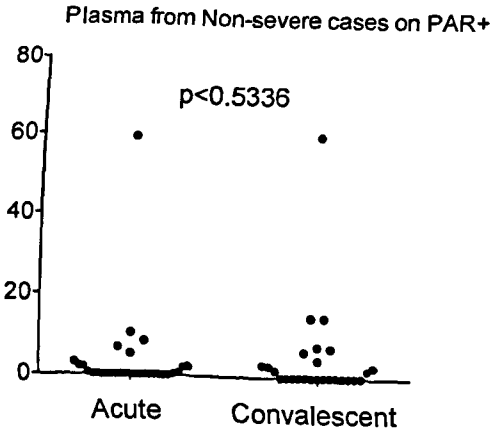
Secondly, analysis was done to compare acute and convalescent antibody responses specifically for samples that were classified to have a single syndromes. Data from 1994-1996 had 12 IC cases and 10 SMA cases while data from 2005-2010 had 30 pure IC cases and 16RD cases. It is important to note that SMA cases and some IC cases may have undergone blood transfusion which could confound their true antibody response as has previously been reported by Bull and colleagues (Bull, Lowe et al. 1999). Figure 6.11and 6.12 shows the acute versus convalescent IgG responses for specific severe syndromes versus the non-severe controls for 1994-1996 and 2005- 2010 datasets respectively.

Between 1994-1996, there was no difference between the acute and convalescent antibody responses in cases with pure syndromes and the non-severe controls (Figure 6.11). This was evident in all the four parasite lines that were tested (Panel A-D). Equally, for the 2005-2010 data, the acute and convalescent antibody responses in with pure syndromes and the controls were not significantly different (Figure 6.12). However, there was a significant difference in the acute-convalescent response in the non-severe group against SA075 parasites. (Figure 6.12, Panel B).

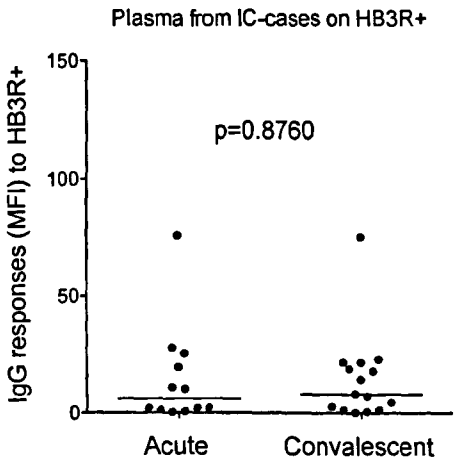
A

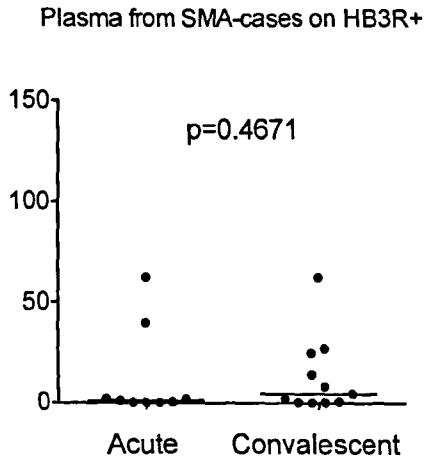

Plasma from IC-cases on PAR+
IgG responses (MFI) to PAR+
80
60
40
20
0
p<0.5852
Acute
Convalescent

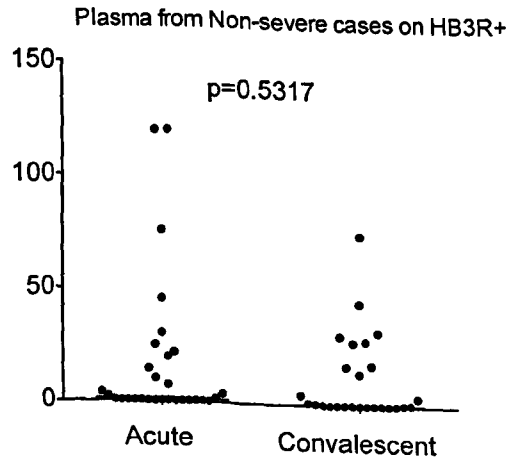

Plasma from SMA-cases on PAR+
80
60
40
20
0
p<0.5401
Acute
Convalescent


Plasma from Non-severe cases on PAR+
80
60
40
20
0
p<0.5336
Acute
Convalescent

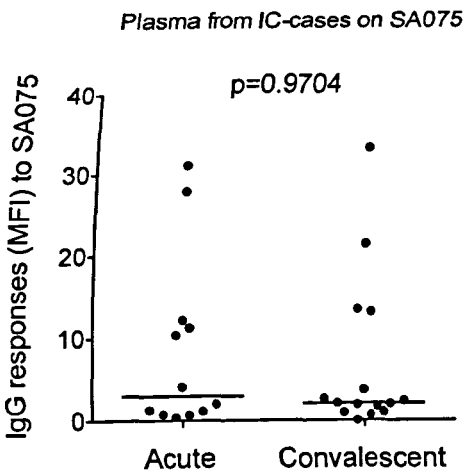
B

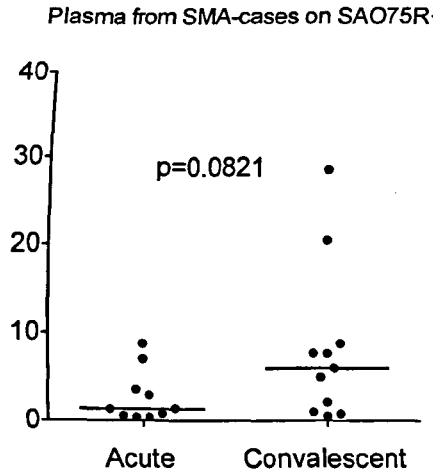

Plasma from IC-cases on HB3R+
IgG responses (MFI) to HB3R+
150
100
50
0
p=0.8760
Acute
Convalescent

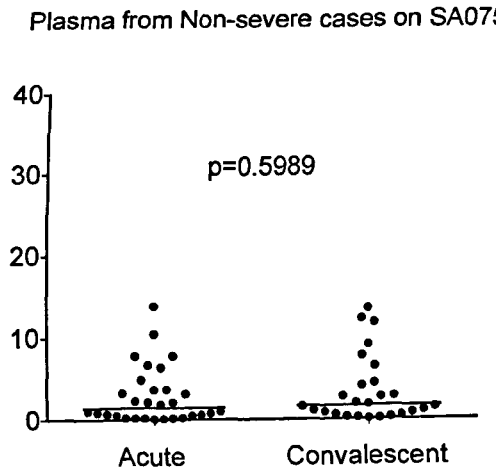

Plasma from SMA-cases on HB3R+
150
100
50
0
p=0.4671
Acute
Convalescent


Plasma from Non-severe cases on HB3R+
150
100
50
0
p=0.5317
Acute
Convalescent

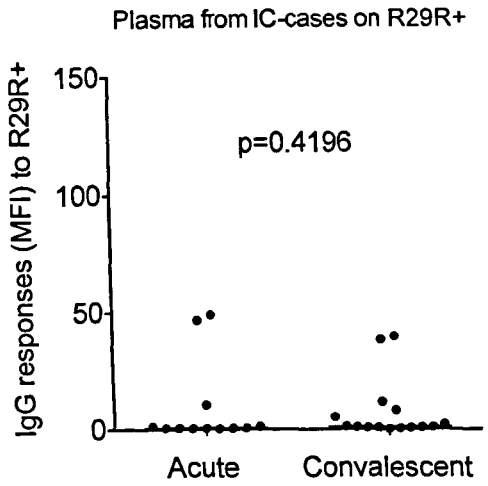
C

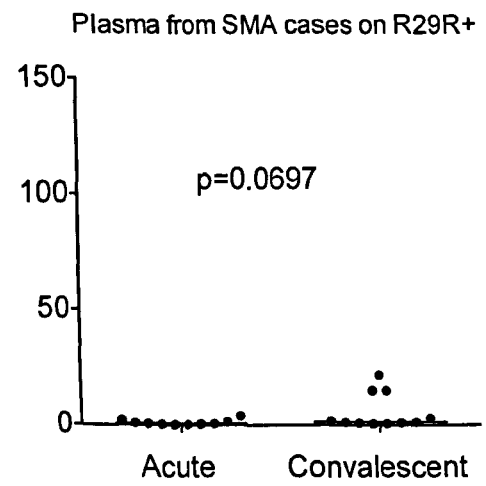

Plasma from IC-cases on SA075
IgG responses (MFI) to SA075
p=0.9704
40
30
20
10
0
Acute
Convalescent


Plasma from SMA-cases on SAO75R+
p=0.0821
40
30
20
10
0
Acute
Convalescent


Plasma from Non-severe cases on SA075
p=0.5989
40
30
20
10
0
Acute
Convalescent

D


Plasma from IC-cases on R29R+
IgG responses (MFI) to R29R+
p=0.4196
150
100
50
0
Acute
Convalescent


Plasma from SMA cases on R29R+
p=0.0697
150
100
50
0
Acute
Convalescent

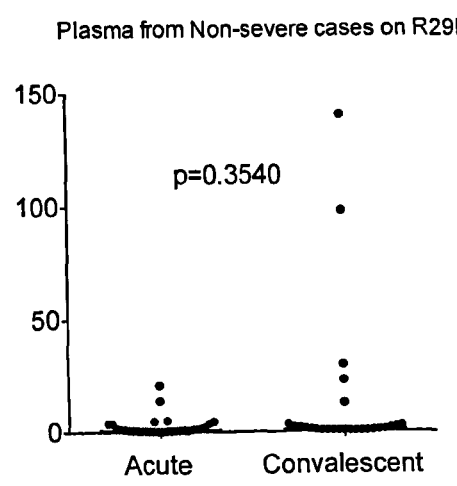
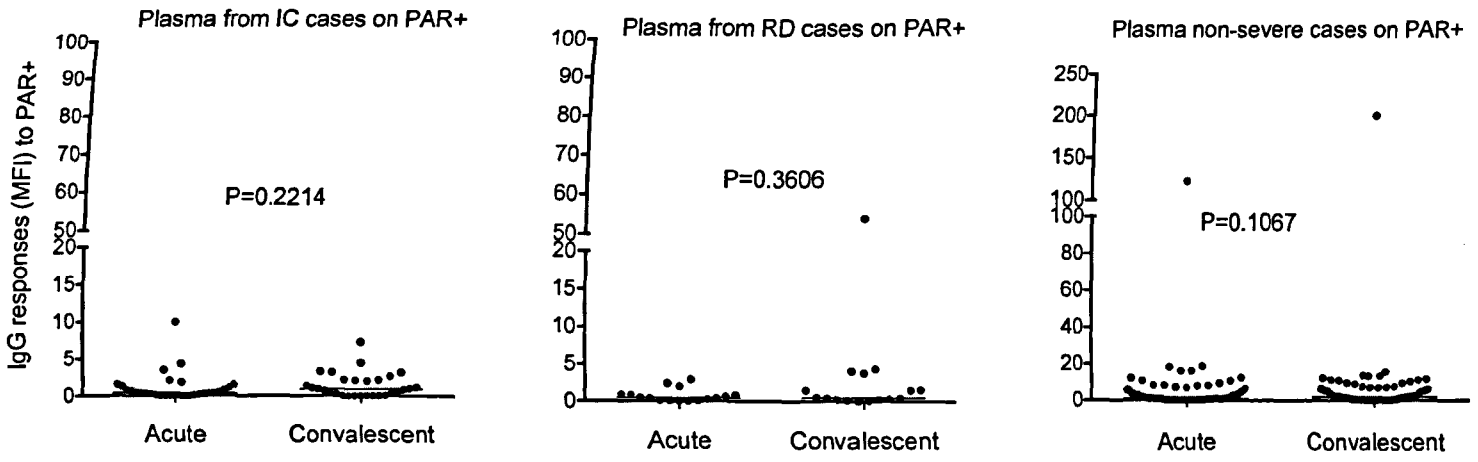

Plasma from Non-severe cases on R29R+
p=0.3540
150
100
50
0
Acute
Convalescent

Figure 6.11: *Data from 1994-1996 showing surface recognition of cell sorted parasites lines by acute and convalescent plasma pairs from children who presented with impaired consciousness (IC), severe malaria anaemia (SMA) and non-severe malaria controls. Shown on the graph are the median of the MFIs with each data point representing plasma from one patient. The parasites tested were A) PAR+ B) HB3R+ C) SA075 and D) R29R+ (Mann Whitney U test, $P<0.05$)*

A


Plasma from IC cases on PAR+
IgG responses (MFI) to PAR+
P=0.2214
Acute
Convalescent
Plasma from RD cases on PAR+
P=0.3606
Acute
Convalescent
Plasma non-severe cases on PAR+
P=0.1067
Acute
Convalescent

B

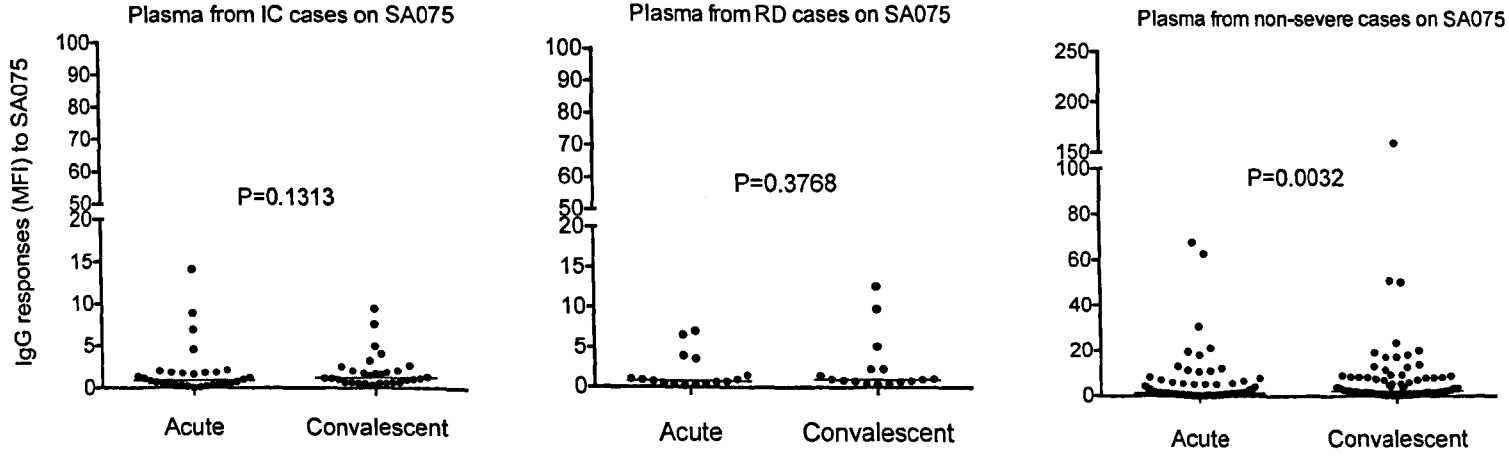

Plasma from IC cases on SA075
IgG responses (MFI) to SA075
P=0.1313
Acute
Convalescent
Plasma from RD cases on SA075
P=0.3768
Acute
Convalescent
Plasma from non-severe cases on SA075
P=0.0032
Acute
Convalescent

Figure 6.12: *Data from 2005-2010 showing surface recognition of cell sorted parasites lines by acute and convalescent plasma pairs from children who presented with single syndromes of impaired consciousness (IC), respiratory distress (RD) and non-severe malaria controls. Shown on the graph are the median of the values with each data point representing plasma from one patient. The parasites used were A) PAR+ and B) SA075 (Mann Whitney U test, $P<0.05$)*

6.4.5: Trends in antibody responses to rosetting parasites between 2004 and 2010

Malaria transmission in Kilifi has been declining lately as evidenced by the declining prevalence in parasitaemia, increased mean age in malaria admissions and a shift in presentation of severe disease (O'Meara, Bejon et al. 2008). This could have had an effect on host-parasite interaction and development of immunity against malaria antigens. I therefore sought to investigate whether the decline in malaria transmission observed in Kilifi over the last 20 years had an effect on antibody responses to VSAs like *Pf*EMP1. To allow for a comparison between the two time periods analysis was done only on PAR+ and SA075 parasites. In this analysis, acute antibody responses by both severe and non-severe cases within each year were pooled together. There was a significant difference in antibody responses to PAR+ between 1994-2010 (Kruskal Wallis P=0.0254) (Figure 6.13 A). On the other hand, there was no difference in the median antibody responses to SA075 over the same period (Kruskal Wallis P=0.2299) (Figure 6.13 B).

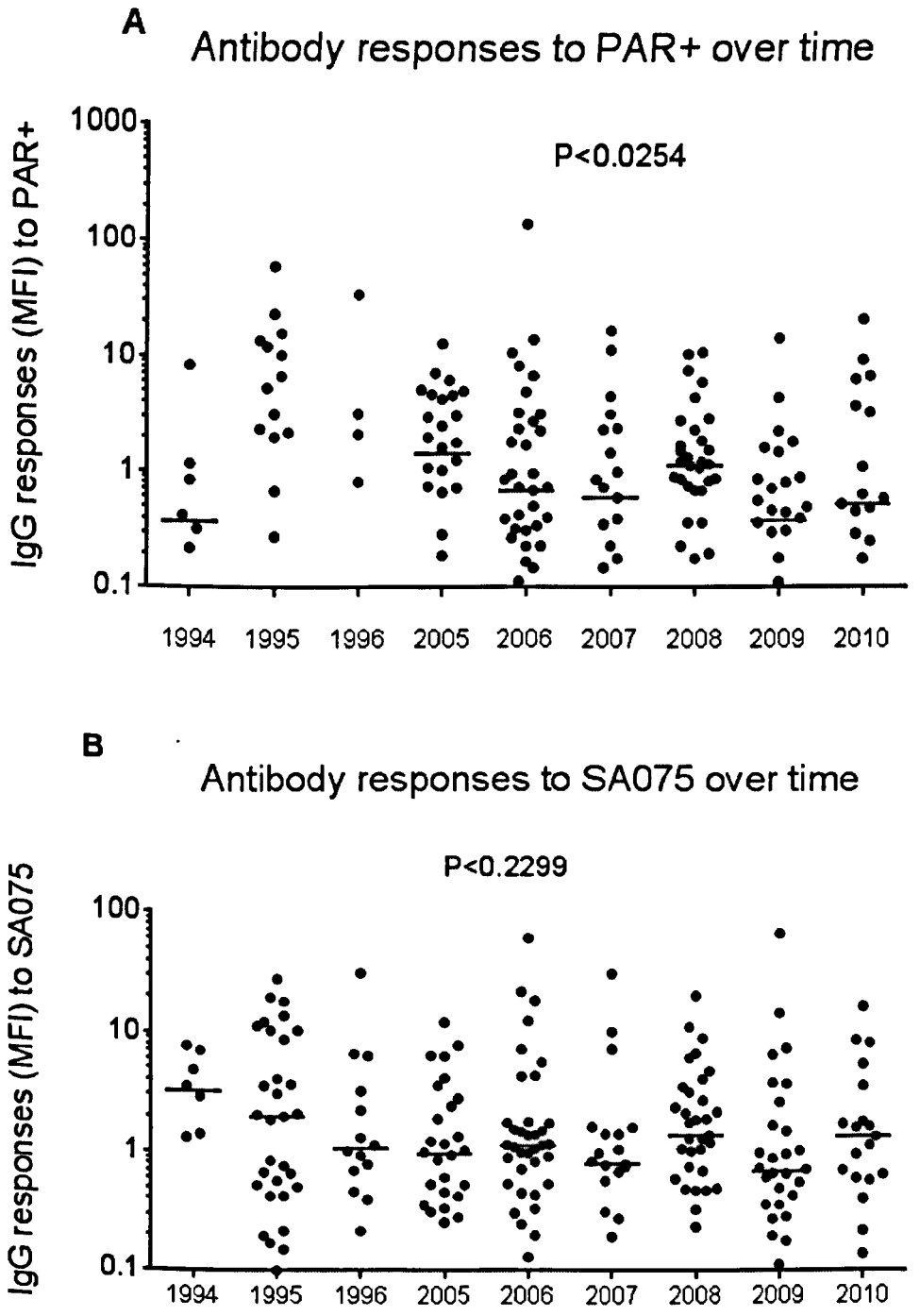
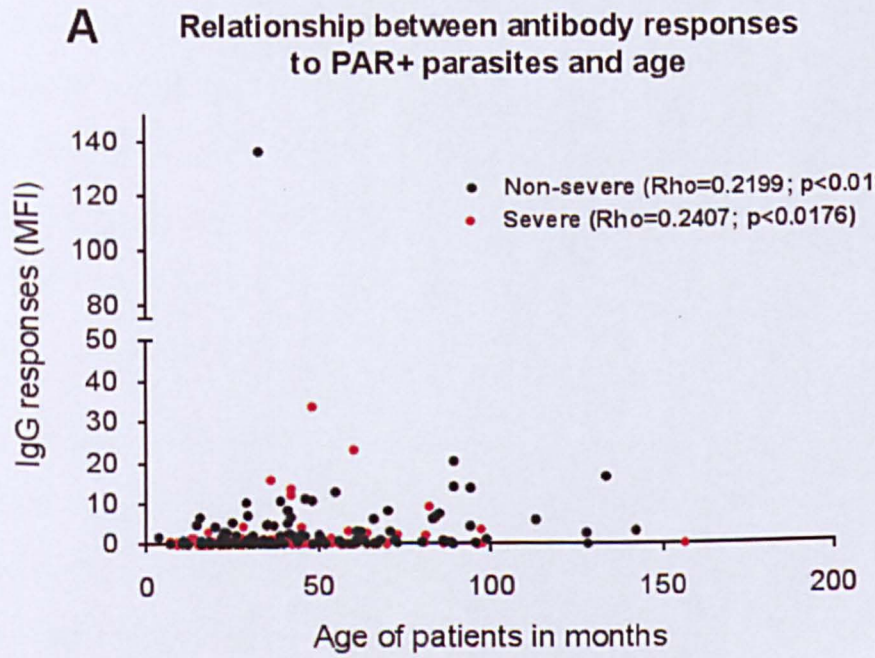


Figure 6.13: *Temporal antibody responses to A) PAR+ and B) SA075 between the years 1994-2010. The dot plots show the median of log transformed antibody*

responses by both severe and non-severe patients with each dot representing an individual patient. Analysis was done by Kruskal Wallis test at P<0.05.

Since age is an important factor in the development of immunity to malaria antigens, the relationship between antibody responses against the two parasites lines and age was also examined for all samples between 1994-2010. In severe malaria cases, only antibody responses to PAR+ and not SA075 were significantly associated with age (Rho=0.2407, P=0.0176) and (Rho=0.1263 P=0.2148) respectively (Figure 6.14 A and B). However, there was a significant positive correlation in antibody responses to both PAR+ and SA075 parasites with age by non-severe cases (Rho=0.2199, P=0.0182) and (Rho=0.3339, P=0.0003) respectively.



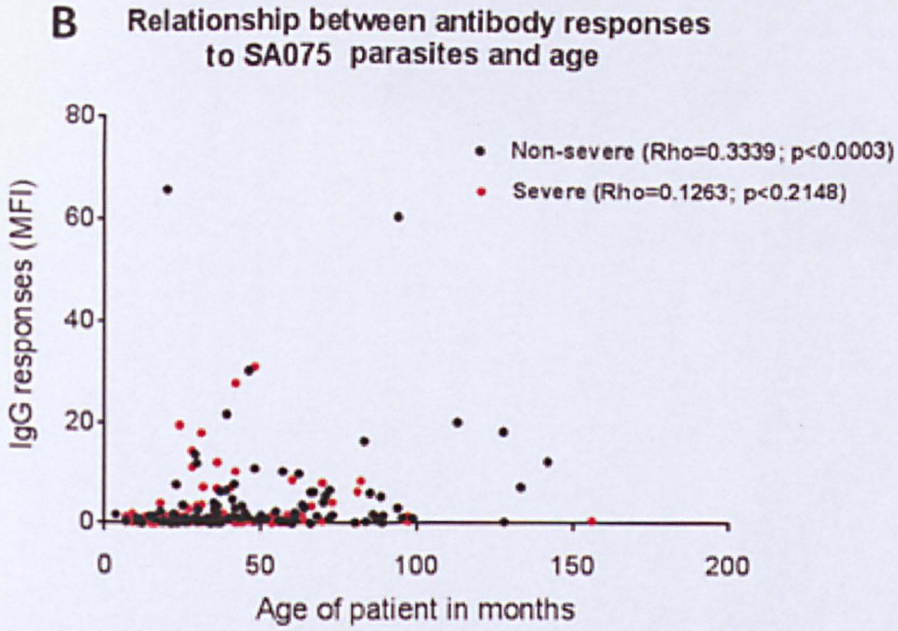


Figure 6.14: *Relationship between acute antibody responses to A) PAR+ and B) SA075 with age. Non-severe cases are represented in black dots while the severe cases were represented with red dots. Spearman's rank test was done to determine strength of the association. Shown on the graphs are correlation coefficients (Rho) and p value. Both severe and non-severe antibody responses against PAR+ were positively correlated with age. However for SA075, only responses to non-severe and not severe cases were positively correlated with age.*

6.4.6: Association between antibody responses and rosetting: Analysis on 48 samples with rosetting frequency data

To further investigate the role of antibodies to rosette mediating variants, analysis was narrowed down to those samples that had rosetting frequency scores. Of the 163 paired samples from the 2005-2010 data set, 48 (29.4%) had rosetting frequency scores ranging from (0-86.2 %). The 1994-1996 dataset did not have rosetting information and therefore was not included in this analysis.

First, rosetting frequencies (% RF) were compared between all the severe cases versus the non-severe cases as has been done in previous studies (Rowe, Obeiro et al. 1995; Newbold, Warn et al. 1997; Heddini, Pettersson et al. 2001; Rowe, Shafi et al. 2002). Although there was a trend towards lower rosetting frequencies in non-severe cases compared to the severe cases, the difference was not significant. (Mann Whitney U test, P=0.1572) (Figure 6.15). Analysis of % RF between the sub-categories of severe malaria was not done due to the small number of samples in each category.

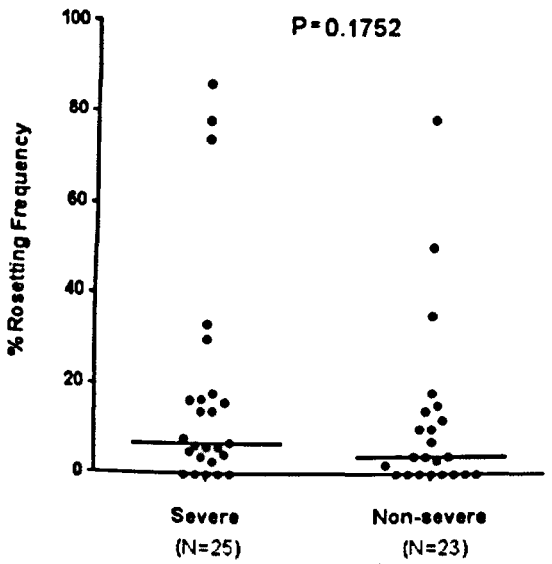
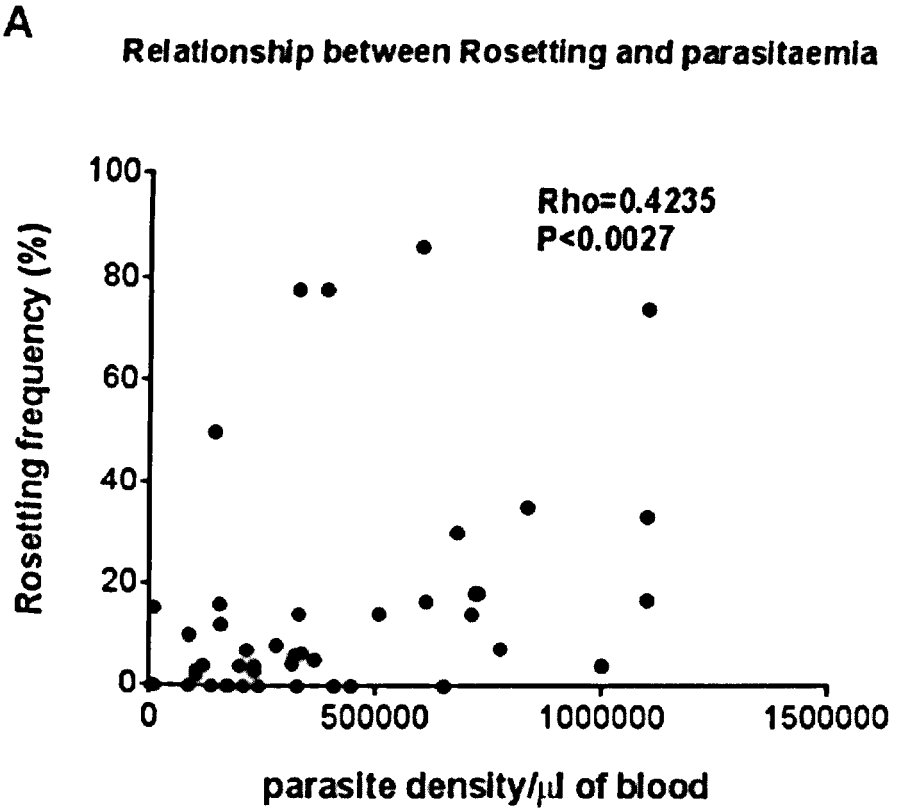


Figure 6.15: *Rosette frequencies of 48 isolates from children with severe and non-severe malaria. There was no significant difference between the %RF in severe versus non-severe cases (P=0.1752).*

Secondly, analysis was done to investigate the correlation between rosetting frequencies with parasitaemia and with age, two factors that are important in development of immunity to malaria antigens. Previous studies have shown positive correlation between rosetting frequency and parasitaemia in samples from

Kilifi (Rowe, Obiero et al. 2002; Warimwe, Fegan et al. 2012). Consistent with these results, there was a significant positive correlation between rosetting and parasitaemia (Rho=0.4235, P=0.0027) (Figure 6.16A). However, there was a tendency towards a negative, although non-significant correlation between rosetting and age (Rho=-0.2307, P=0.1188) (Figure 6.16B).

A



B

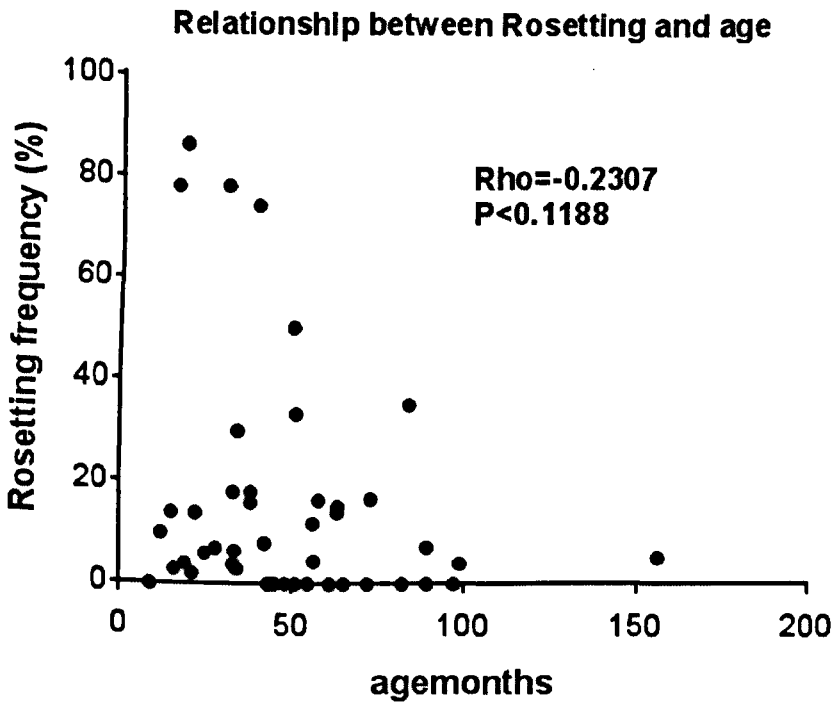


Figure 6.16: *Relationship between rosetting frequency (% RF) with A) parasitaemia and B) age using the 48 samples that had rosetting scores. There was a significantly positive correlation between % RF with parasitaemia (Rho=0.4235, P=0.0027) but not with age (Rho=-0.2307, P=0.1188).*

Thirdly, acute and convalescent antibody responses against PAR+ and SA075 were assessed using the 48 samples. While the acute versus convalescent responses in PAR+ were not significantly different, there was a significant difference in SA075 responses (Figure 6.17). Results on the entire 2005-2010 dataset (regardless of whether they had rosetting data or not) described in section 6.4.4 had shown a significant difference in acute-convalescent response against SA075 only in the non-severe patients. Of the 48 samples, the non-severe cases represented 50% of these samples and that could partly explain the similarity between responses to SA075 in this data and the entire 2005-2010 dataset.

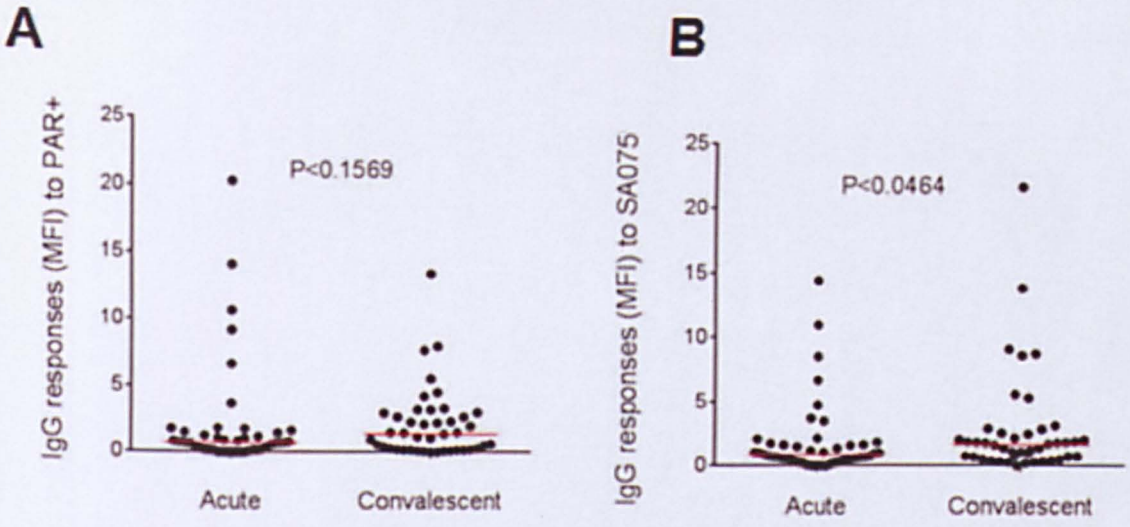


Figure 6.17: *Surface recognition of A) PAR+ and B) SA075 using the 48 acute and convalescent plasma pairs from children whose rosetting frequencies had been recorded in between 2005-2010. Shown on the graph are the median of the MFIs with each data point representing plasma from one patient (Mann Whitney U test, P<0.05).*

Further analysis was done on the 48 samples to examine the association between rosetting and antibody responses to PAR+ and SA075. These results would highlight whether epitopes within these two parasites were similar to those of rosetting parasites occurring frequently in natural populations. Separate scatter plots were made for the acute and convalescent responses (Figure 6.18). Both the acute and convalescent antibody responses to PAR+ had a significant negative correlation with rosetting frequency of the isolates from which the children's plasma samples were obtained, acute (Rho=-0.232, P=0.0316) and convalescent (Rho=-0.3043, P=0.0472). SA075 showed a significant negative correlation between antibody responses and rosetting frequency at acute phase but not at

convalescent phase, acute (Rho=-0.3198, P=0.0027), convalescent (Rho=-0.0083, P=0.9404).

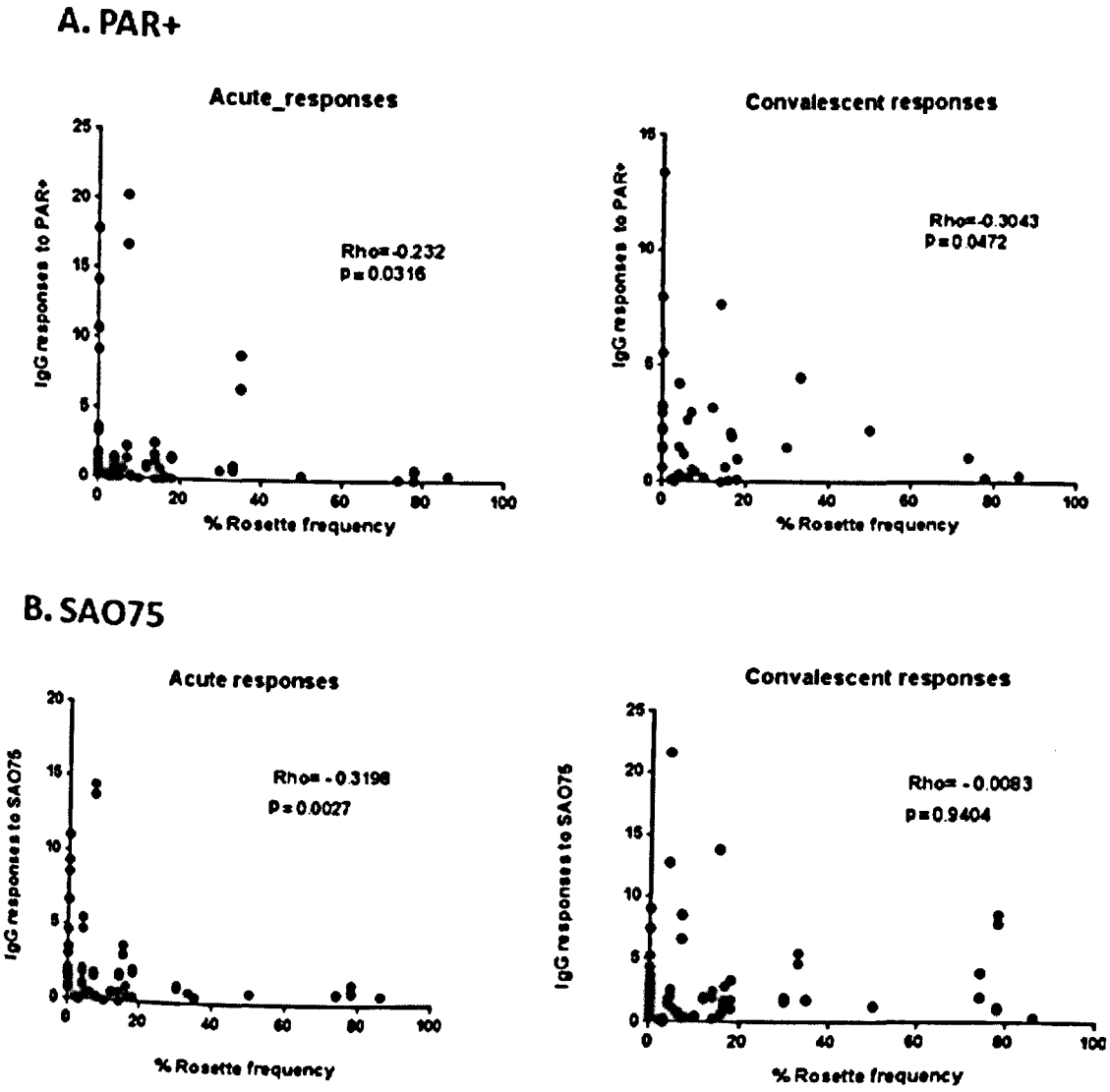


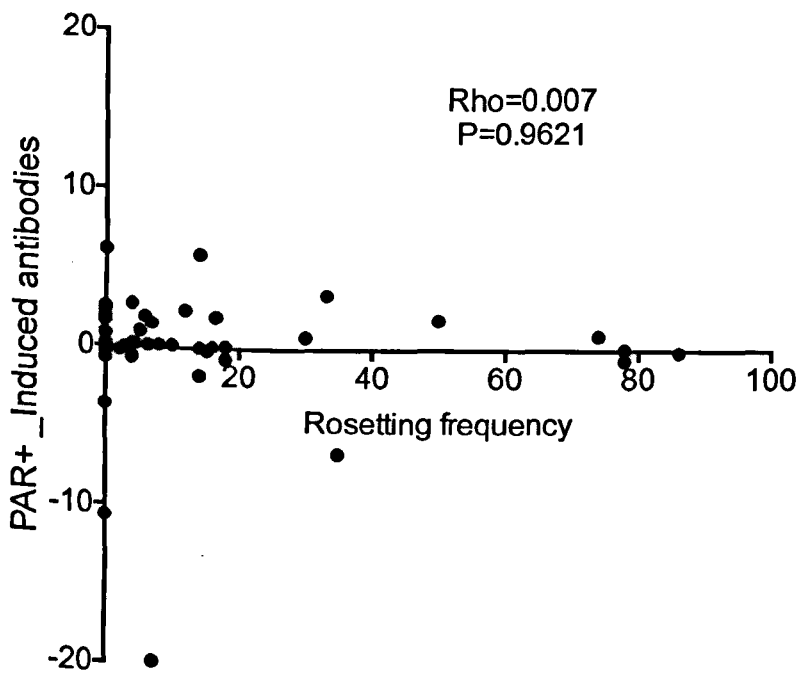
Figure 6.18: Relationship between rosetting frequency with acute and convalescent antibody responses in A) PAR+ and B) SA075 using the 48 samples that had rosetting scores. There was a significant negative correlation between % rosetting frequency and acute (Rho=-0.232, P=0.0316) and convalescent (Rho=-0.3043, P=0.0472) antibody responses in PAR+. In SA075, there was a negative

correlation only at acute stage (Rho=-0.3198, P=0.0027) but not at convalescent stage (Rho=-0.0083, P=0.9404).

Further analysis was done to examine whether the induced antibody responses (the difference between acute and convalescent responses) correlated with rosetting frequency. A positive correlation would suggest that the antibody responses were targeting parasite epitopes similar to those whose rosetting frequency was taken. This would be a further confirmation that the rosetting parasite being tested was common within the population. Figure 6.19B shows a significant positive correlation between responses to SA075 and rosetting frequency (Rho=0.2872, P=0.0478). This was not the case for PAR+ (Rho=0.007, P=0.9621) (Figure 6.19A).

A

PAR+ Correlation between Induced Antibodies and % RF



B

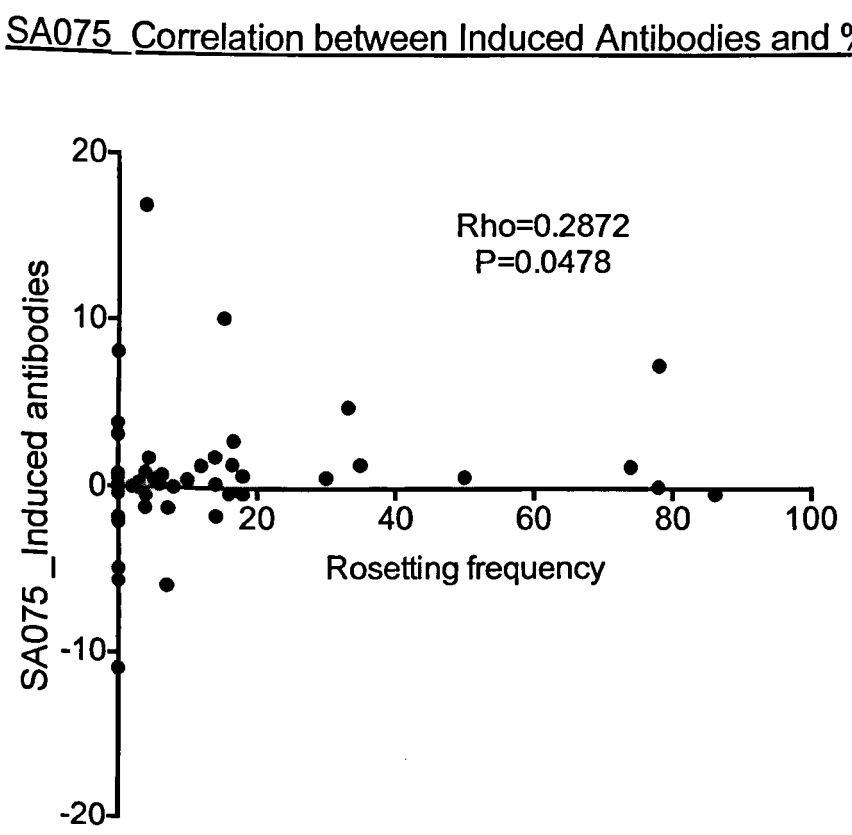


Figure 6.19: *Correlation between induced antibody responses to A) PAR+ and B) SA075 against rosetting frequency*

6.5 DISCUSSION

Although *Pf*EMP1 has generally been implicated in pathogenesis of severe malaria, the interactions responsible for causing specific disease syndromes are poorly understood. Additionally the manner in which host immunity i.e. anti-*Pf*EMP1 antibodies are able to modulate the clinical outcome of malaria is not clear. There was need for more studies to explore the clinical importance of different *Pf*EMP1 variants. In this study, the clinical relevance of rosette-mediating variants was examined in Kilifi, Kenya.

To achieve this objective, cell sorted parasites that were enriched to express single variants were used. This was important because one major difficulty in studies on antibody responses to native protein on the surface of infected RBCs has been the rapid antigenic variation of *Pf*EMP1 which potentially results in non-homogeneity in the surface-displayed molecule when parasites are put in culture. To overcome this, different parasites lines have been selected using adhesive molecules or antibodies specific to certain *Pf*EMPI variants in order to generate parasites enriched in a single variant. More recently, studies were carried out on HBEC-5i and HBMEC selected parasites to examine the role of these molecules in cerebral malaria (Avril, Tripathi et al. 2012; Claessens, Adams et al. 2012). Similar studies have been carried out on panned VarO expressing rosetting parasites using plasma from children in West Africa (Vigan-Womas, Lokossou et al. 2010).

In this study, the parasites were cell sorted using their homologous NTS-DBLα antibodies (Chapter 5). The enriched parasites were tested against acute-convalescent plasma pairs from children with different clinical syndromes of malaria. Studies have previously been carried out in Kilifi to explore the kinetics of antibody responses to VSAs using acute and convalescent clinical samples either by FACS or agglutination assays (Bull, Lowe et al. 1999; Kinyanjui, Bull et al. 2003). However, none of these studies focused specifically on rosetting-mediating variants as was done in this study.

In the matched case-control study described in this chapter, results showed an induction of antibodies to PAR+ and HB3R+ parasites in patients with CM and RD respectively (Figure 6.7 and 6.8). This would suggest that *Pf*EMP1 variants expressed by the patients' parasites at the time of disease were antigenically

similar to PAR+ and HB3R+. A recent study by Ghumra *et al.*,2012 showed the existence of shared surface epitopes amongst rosette-mediating variants from parasites with IgM-binding phenotype. Interestingly, both PAR+ and HB3R+ parasite have the IgM binding phenotype. However, the IgM binding phenotype for the field isolates whose plasma samples were used was not established. It can only be speculated that they had the IgM phenotype hence generation of cross reactive antibodies. This may be supported by results from a previous study by Rowe *et al.*, (Rowe, Shafi et al. 2002) in which clinical isolates in Kilifi, Kenya showed a positive link between IgM binding, rosetting and severity of disease.

Although both PAR+ and HB3R+ have the IgM-binding phenotype, they have differences at the phenotype and sequence level as well as their geographical origins. PAR+ is a knobless parasite (Udomsangpetch, Aikawa et al. 1989) originally from S.E Asia while HB3R+, which is originally from Honduras, has knobs. At the sequence level, PAR+ falls into the CP6 of Cys/PoLV classification (Bull, Berriman et al. 2005) while HB3R+ belongs to CP3. Both however belong to block sharing group 1 (BS1) which is strongly linked to Group A-like sequences (Bull, Buckee et al. 2008). Based on domain cassette classification, PAR+ belongs to DC11 with a DBLα 1.8 sub-type while HB3R+ belongs to DC16 with a DBLα 1.5 sub-type (Ghumra, Semblat et al. 2012). In the study by Ghumra (Ghumra, Semblat et al. 2012), the few clinical isolates that were studied showed surface reactivity with either antibodies to DBLα1.5 (domain cassette 16) or DBLα1.8 (domain cassette 11), but rarely with both. It is possible therefore that the differences between the two parasite lines could explain their differential association with CM and RD.

It was interesting, however, to observe an induction of antibodies against some parasites in plasma from uncomplicated malaria cases. The responses by the RD controls against TM284R+ and R29R+ (Figure 6.8B and D) as well SA075 in the population-based study (Figure 6.12 B) all showed significant induction of antibodies when comparing acute versus convalescent. None of these parasites was associated with severe disease in this study. Two possible explanations could be given for this observation; 1) that some rosette mediating *Pf*EMP1 variants are not directly associated with severe disease. A recent study by Ghumra et al 2012 (Ghumra, Semblat et al. 2012) seems to suggest that rosetting parasites with the IgM-binding phenotype seem to play a key role in pathogenesis of severe disease, evidence that was supported by the positive association between rosetting, IgM binding phenotype and severe disease in clinical samples in a previous study (Rowe, Shafi et al. 2002). This fact may however be overruled by the fact that TM284R+ also binds non-immune IgM. 2) It could also be that apart from the mere presence of rosettes, the size and strength of the rosettes play a role in development of clinical disease. Host genetic factors are also important in determining the clinical outcome. These features were however not evaluated in this study.

Due to the limited number of samples in the case-control study, analysis was expanded to include more plasma samples collected between 1994-1996 and 2005-2010. This analysis however included children with overlapping syndromes of malaria and the pure cerebral malaria-CM cases were included in the larger impaired consciousness-IC category. Additionally, the analysis involved severe malarial anaemia-SMA cases whose antibody responses may have been confounded by blood transfusion (Bull, Lowe et al. 1999). Apart from examining

the induction of antibodies between acute and convalescent time-points, data from this study were also used to examine temporal antibody responses over time as well as relationship between rosetting and parameters associated with immunity such as age and parasitaemia.

By pooling all the samples with a clinical syndrome into one category of "severe" cases, antibody responses against R29R+ showed a significant difference between the acute and the convalescent in the severe group but not in the non-severe group (Figure 6.9). None of the other parasites exhibited a significant difference during the 1994-1996 time period. However, in the 2005-2010 data set, antibody responses to PAR+ were significantly higher in the convalescent than in the acutes in the severe cases but not the non-severe cases (Figure 6.10). This effect could have been strongly driven by the CM cases who showed similar kinetics against PAR+ in the case-control study. In SA075, there was a significant diference in the acute and convalescent responses in both the severes and the non-severes. However, when the severe cases were broken down into single pure syndromes, none of the severe malaria syndromes exhibited this difference between the acute and convalescent (Figure 6.11 and 6.12). The differences in the trends of antibody responses against the same parasite between the two time periods could perhaps be due to differences in malaria transmission observed between the two time periods in Kilifi (O'Meara, Bejon et al. 2008). An analysis of the *var* gene expression profiles during these two periods would highlight whether there are changes that could explain the differences seen in this study.

Immunity to malaria has been shown to build up with age and has been shown to be protective against clinical malaria (Langhorne, Ndungu et al. 2008; Doolan,

Dobano et al. 2009). To examine whether antibodies targeting rosette-mediating variants also follow a similar pattern, IgG responses to PAR+ and SA075 from 1994-2010 were correlated with age. There was a significant positive association between antibodies to PAR+ and age in both the severe and non-severe cases (Figure 6.14 A.). SA075 also showed an overall positive correlation although it seemed to be limited to the non-severe cases and not in the severe cases (Figure 6.14B). The results suggest that these antibodies also play a key role in the age dependent-acquisition of immunity to malaria. Based on the case control results where PAR+ seemed to be important in CM, the age build-up of immunity to PAR+ seems to be important in modulating CM.

Analysis was then focused on 48 samples that had rosetting scores taken during the first trophozoite stage in culture. The % RF in these samples ranged between (0-86.2%). A comparison between the severe and non- severe cases showed no difference in the rosetting frequency between the two groups (Figure 6.15). Contrary to this result, previous studies done in Kenya, Gabon and Madagascar have shown a difference between severe malaria and uncomplicated malaria (Ringwald, Peyron et al. 1993; Rowe, Obeiro et al. 1995; Newbold, Warn et al. 1997; Kun, Schmidt-Ott et al. 1998; Heddini, Pettersson et al. 2001; Rowe, Shafi et al. 2002). The differences between the current results and those previously published could be due to the definition of the control group. In this study non-severe group encompasses both mild malaria and moderate malaria both of which have in a previous study shown differences in their rosetting frequencies when compared to the severe cases (Rowe, Obeiro et al. 1995).

There was a positive association between rosetting frequency and parasitaemia (Figure 6.16A). This was consistent with previous studies done in Kilifi (Rowe, Obiero et al. 2002; Warimwe, Fegan et al. 2012). The role of rosetting in the pathogenesis of severe disease is thought to be the obstruction of microvascular blood flow resulting into tissue hypoxia and organ damage (Kaul, Roth et al. 1991; Dondorp, Ince et al. 2008). It is also thought to result in increased growth rates caused by quick invasion of merozoites into the non- infected cells in rosetting parasites resulting to high parasitaemia, although an *in vitro* experiment showed contrary results (Deans and Rowe 2006). There was a negative (although non-significant) association between rosetting frequency and age. Age is an important factor in host pathogen interactions and clinical manifestations of severe disease tend to occur in children of different age-groups (Marsh, Forster et al. 1995). Cerebral malaria, which has been associated with rosetting in some studies (Carlson, Helmby et al. 1990; Treutiger, Hedlund et al. 1992; Rowe, Obeiro et al. 1995), has been shown to occur in older children (Newton and Krishna 1998). Such studies would thus support a negative association between rosetting and age as was seen in this study.

Based on the 48 samples, there was no increase in variant-specific antibody responses to PAR+ at acute versus convalescent time points. However, there was a significant induction of variant specific responses to SA075 between the two time points (Figure 6.17 Panels A and B). The data on correlation between rosetting frequency and antibody responses to PAR+ and SA075 (Figure 6.18) is intriguing and supports data in Figure 6.17. The negative association between acute antibody responses against PAR+ and % RF was maintained even at convalescence suggesting that there was no buildup in antibodies against PAR+

(Figure 6.18A). In SA075 however, there was a negative association at acute stage which was lost at convalescence suggesting that there was a change in response at the two time points (Figure 6.18B). Further analysis (Figure 6.19) shows a significant positive correlation between the induced antibody responses and rosetting frequency in SA075 but not in PAR+. These results would suggest in part that rosetting parasites similar to SA075 were common in natural populations. However, based on the case-control data, such variants seem not to be associated with any clinical syndrome. PAR+ on the other hand may be a less common rosetting parasite in the population although its presence is associated with cerebral malaria in the case-control study. It could be that the common VSAs circulating within the population are not necessarily the virulent ones. Whether some rosette-mediating variants are clinically more important than others needs to be explored further.

There were several shortcomings in this study. 1) *Var* gene expression profiles were not examined for the parasites whose plasma were used in the analysis. A study by Warimwe and collegues (Warimwe, Keane et al. 2009) showed how expression of *var* genes bearing two-cysteine residues in the DBLα tag region (Cys2 *var* genes) were associated with severe malaria and young host age. Additionally, the data showed that *var* gene expression was differentially associated with severe malaria syndromes (Warimwe, Keane et al. 2009; Warimwe, Fegan et al. 2012). Although the *var* gene expression profiles for all the rosette-mediating variants used in this study were known (Chapter 3 Table 3.1), the expression profile for the patients' parasites may have been used to explain some of the associations observed in this study. 2) While the presence of variant specific antibodies may be crucial in understanding the clinical relevance of the

rosetting variants, the exact role of the antibodies in modulating severe disease remain unknown. Field studies by Carlson *et al.,* 1990 (Carlson, Helmby et al. 1990), Barragan *et al.,* 1998 (Barragan, Kremsner et al. 1998), and Vigan-Womas *et al.,*2010 (Vigan-Womas, Lokossou et al. 2010) showed that the mechanism of protection by antibodies to rosetting variants could be either cytophilic or their ability to disrupt rosettes. In the current study, the role of the anti-rosetting antibodies was not evaluated. 3) Exposure and multiplicity of infections (MOI) seem to be important confounders in most studies on antibody responses to malaria antigens. Despite the fact that patients came from the same geographical location and were of the same age, some may be more exposed to infections than others thus increasing their antibody repertoire to malaria VSAs including *Pf*EMP1 variants. Schizont extract ELISA has consistently been used as a proxy measure of exposure. In the current study, the ELISA was not done.

In summary, these data show that rosette-mediating *Pf*EMP1 variants may play a role in clinical malaria. PAR+ and HB3R+ are rosetting strains whose variants seem to be linked to cerebral malaria and respiratory distress. SA075 on the other hand is a parasite whose variant was not directly linked to any of the syndromes of severe malaria but SA075*var*1-like variants may be common in the parasite population in Kilifi. Addressing some of the limitations of this study may however provide stronger evidence to support these findings.

CHAPTER 7

Conclusion & Recommendations

The main focus of this thesis was to further understand the molecular mechanisms of rosetting, particularly in field isolates by characterizing the *Pf*EMP1 variants and to investigate the clinical relevance of rosette-mediating variants. This was crucial considering that rosetting has consistently been associated with severe malaria in African children. Secondly, association studies do not necessarily show a causal relationship and therefore there still remains a gap that directly links rosetting to any of the syndromes of severe malaria. The aim therefore was to identify and characterize a rosette-mediating *Pf*EMP1 variant from a Kenyan field isolate and to examine the profiles of antibody responses against the rosette-mediating variants in a Kenyan population exposed to malaria. In the preliminary result chapter, the inability to identify *var* genes associated with rosetting highlights the need to carry out a more systematic identification and characterization of *Pf*EMP1 variants in field isolates. However, the subsequent successful identification and sequencing of a full length *Pf*EMP1 variant from one of the field isolates and the ability to induce functional anti-*Pf*EMP1 antibodies is crucial in the development of *Pf*EMP1 vaccines. A key finding reported in this thesis was the ability of patients recovering from severe malaria to raise variant-specific antibody responses against some rosette-mediating variants. These data demonstrates the clinical relevance of these variants by linking them to particular severe malaria syndromes, thus making them important targets for vaccine development. Results also showed that some *Pf*EMP1 variants associated with rosetting may be common in natural populations although they may not necessarily be associated

with severe disease. More studies need to be done on functional and immunological characterization of rosette-mediating variants from field isolates.

In Chapter 3, I attempted to identify *var* genes associated with rosetting from 4 culture adapted field isolates from Kilifi. However single dominant genes linked to rosetting were not identified in the transcript profiles of the five field isolates. Although the original objective was to monitor the transcription profiles in the course of the rosette selection, it is possible that the selection method was not efficient enough and that the differences in clones between the acute and the rosette selected samples based on MSP1 and MSP2 genotypes contributed to the lack of identification of the rosette-mediating gene. A better approach would be to obtain highly rosetting isolates from the field and examine their transcript profiles.

In Chapter 4, a putative rosette-mediating gene, SA075*var*1 from a field isolate called SA075 was identified and characterized. The DBLα tag sequences for the SA075 *var* gene had been previously characterized (Bull, Buckee et al. 2008). It had interesting sequence features such as belonging to Cys/PoLV group 2, block sharing group 2 and sequence signature 2 (sig2) that have all been previously linked to rosetting (Bull, Berriman et al. 2005; Bull, Buckee et al. 2008). This made SA075 an interesting parasite to study. Unlike in Chapter 3 where rosette selection on field isolates were done by flotation method prior to transcript profiling, analysis of SA075 transcripts were done following cloning by limiting dilution. The same dominant *var* gene that was identified in the previous characterization (Bull, Buckee et al. 2008) was identified in the rosette positive clone. Despite a long and iterative process of PCR walking, cloning and sequencing, the full-length gene was successfully cloned and sequenced. The whole genome sequence of SA075

isolate is being assembled at the Sanger Institute and the next task will be to compare the SA075*var*1 sequences as generated by the two methods. Recent classification of *Pf*EMP1 by domain cassettes (DCs) (Rask, Hansen et al. 2010) showed that SA075*var*1 had DC8-like features. This makes SA075 *var*1 to be of interest considering that DC8 has lately been linked to severe malaria (Lavstsen, Turner et al. 2012; Bertin, Lavstsen et al. 2013).

In Chapter 5, the ability of SA075*var*1 to induce functional antibodies was examined. However, one major finding during characterization of these antibodies was the possibility that the SA075*var*1 gene was not involved in rosetting of SA075 parasites. There were inconsistencies between the % RF by microscopy and % positive population by flow cytometry following staining by anti-SA075*var*1 in both cell sorted and unsorted parasites. Microscopy is open to inter-observer variation, and therefore could have been a huge contributor to this discrepancy. The strength of rosettes which was not examined in this thesis may also have contributed. Loose rosettes which could be easily disrupted would easily result in low % RF and high % positive population as was mostly the case in SA075. The possibility of a recombination event that may have an effect on antibody responses and the nature of rosettes can also not be ruled out. None the less, assays were carried out that showed an induction of functional SA075*var*1 antibodies. The antibodies had the ability to recognize the native surface of infected RBCs and mediate phagocytosis as has been done in other rosetting strains (Ghumra, Khunrae et al. 2011). More work needs to be done to ascertain whether SA075*var*1 is indeed involved in rosetting in SA075 parasites. This includes a systematic experiment to monitor the correlation between % RF by microscopy and high % positive population by flow cytometry. The DC8-like features of

SA075*var*1 as discussed in Chapter 4 also make it an interesting *Pf*EMP1 variant to study. Although not discussed in this thesis, preliminary work to this end showed that some of the real time primers targeting DC8 (Lavstsen, Turner et al. 2012) were able to amplify transcripts from cDNA of SA075.

Finally in chapter 6, I explored whether rosetting variants had any clinical relevance and whether children recovering from different syndromes of severe malaria raise variant specific antibodies against rosetting parasites. The approach used in this chapter in which acute and convalescent responses were analysed has not been used previously to examine antibody responses against rosette mediating variants and has provided a direct link of rosetting with severe disease in the absence of an in vivo model. Despite the limited number of samples in the case-control study, variant specific antibody responses against PAR+ and HB3R+ suggested that *Pf*EMP1 variants that are antigenically similar to these two could be important for cerebral malaria and respiratory distress respectively. The findings also highlight the presence of cross-reactive antibodies in plasma from children who had severe malaria that had the ability to recognize antigenically similar parasites. Although a study by Doumbo *et al* 2009 (Doumbo, Thera et al. 2009) showed that rosetting was associated with all forms of severe malaria, the differential link by PAR+ and HB3R+ to different syndromes is intriguing and could suggest that there are different types of rosetting variants associated with different syndromes of severe disease. What then are the differences between the two rosetting variants? At the sequence level, PAR+ and HB3R+ parasites belong to different CP groups (6 and 3 respectively) as well as different DC classes (11 and 16 respectively). They do however belong to the same block sharing group 1 and both have the IgM binding phenotype. Although previous studies of rosetting have

shown an association between rosetting and cerebral malaria, the findings on antibody responses to PAR+ by cerebral malaria patients could strengthen the importance of rosetting in development of severe disease. The absence of case-control plasma samples from patients with severe malarial anaemia was a caveat in this analysis since rosetting has in some studies been linked to low haemoglobin levels (Rowe, Shafi et al. 2002). This would also have been particularly interesting for SA075 parasites which were originally obtained from a patient with severe malarial anaemia.

Despite the small sample size in the case-control study, the careful matching of the cases to the controls by age, date of admission and blood group minimized the possible confounding effect by these factors. The population-based study on the other hand had the advantage of a bigger sample size but it is possible the true effect of the variant-specific antibody responses when comparing the cases and controls may have been confounded. The positive results by PAR+ and HB3R+ in the case-control study need ideally be confirmed in a larger study of the same design.

The findings in chapter 6 also pose a question as to whether there are two types of rosetting phenotype: - those that are associated with severe disease (as in PAR+ and HB3R+) and those that are not (as in TM284R+, R29R+ and SA075). Interesting results were further seen with SA075 in which there was a positive correlation between % RF in 48 samples and induced antibody responses against the cell sorted parasites. These results suggested that the variant expressed by this parasite is common within the population despite not previously being associated with any syndrome. This raises the possibility that severe malaria is a

complex phenomenon that cannot be explained by examining antibody responses by the host to one parasite/antigen. *Var* gene expression profiles have also been shown to be modified by host immunity at the time of disease. This aspect was however not explored in this thesis. Other aspects of the study that would have been important if examined were the presence of anti-rosetting antibodies in the plasma of patients. It could be that anti-rosetting activity and not just the presence of the surface reactive antibodies is important in modulating the outcome of the disease.

Overall, the findings from this thesis highlight the heterogeneity of *Pf*EMP1 variants associated with the rosetting phenotype as well as the heterogeneity of severe malaria and suggests that more studies especially on field isolates need to be done in order to fully understand the molecular mechanisms of rosetting.

CHAPTER 8

Appendix

8.1 SSC protocol 1131

APPENDIX 2.1A
Information sheet
(Children recruited at dispensaries or hospital)
Integrated studies of the development of natural immunity to malaria in children in Kilifi district

Lead Investigator: Kevin Marsh, Director KEMRI-Wellcome Programme.

What question is KEMRI trying to answer?
KEMRI is an institution which learns more about diseases to find better ways of preventing and curing those diseases. An example is malaria, which causes much death and illness among Kenyan children. Children are protected against measles by the measles vaccine. Children could be protected against malaria if there was a malaria vaccine. In areas like this people become immune to the bad effects of malaria as they grow older. In KEMRI we are working to try to understand how people become immune to malaria. This information is important in designing vaccines against malaria.

You have come to the dispensary/ hospital today because your child has an illness that may be caused by malaria. We would like to invite you to allow your child to take part in a study to help find out how people become immune to malaria.

What does taking part involve?

Normally your child would receive some blood tests to confirm the problem and then receive treatment. These things will happen in the normal way but I addition we will ask to take an extra sample of blood for research.
If you agree for your child to be part of the study we will:
1. Take a 5mls (one teaspoon) of blood from a vein on your child"s arm or hand.
   i. This will be used for tests that help the doctors decide how to treat your child.
   ii. The remainder will be used for research

2. Ask permission to take a further 5 mls blood sample after your child has recovered from the illness.
   i. This will be approximately three weeks from now.
   ii. We will later invite some children to return at six, twelve and twenty four weeks from now for further blood samples.
   iii. We will make it clear to you whether we are asking you to return only once or a total of four times

Whether you agree to take part or not your child will receive the normal treatment for their illness.

Are there any risks to being in the study?

Taking a blood sample can be uncomfortable and can sometimes cause mild bruising. The amount of blood taken is completely safe, even if your child is anaemic. If your child is anaemic they will receive treatment for this along with treatment for any other illness.

Are there any benefits to being in the study?
We will provide the cost of bus fare for you and your child to attend for the follow up blood sample(s). At that time there will be an opportunity to see a health worker, and have appropriate tests performed, if you have any concerns that your child is not completely recovered. This research may have a long term benefit to children in this community and others in similar communities through providing information that will help in the development of better ways of preventing malaria. Otherwise there are no specific benefits.

What happens to the blood samples taken in the study?

In the laboratory we will examine the blood to see if it can protect against malaria. The ability to protect can be present from birth but can also develop as we grow older.
Most of the tests will be done in the laboratories in Kilifi. Some of the tests cannot be done in Kenya and so will be done in laboratories overseas. Not all the tests will be done at the same time and so the blood will be stored in a freezer until the tests can be done. At the end of the study any blood that has not been used in tests will be stored so that further tests of the kind described above can be carried out in future.

Who has allowed this research to take place?

An independent committee in Kenya that is run by the Government and a committee in Kilifi have looked carefully at this and agreed that the research is important, that it should be safe to take part in and will be conducted properly.

What if I decide not to involve my child or change my mind later?

Participation in research is completely voluntary. Nobody is obliged to take part and anyone who does agree can change their minds at any time. If you refuse to take part, or agree and alter change your mind there are no penalties what so ever, and you and your child will still be able to benefit from the improved level of service offered at the dispensary and at Kilifi hospital as a result of the research carried out by KEMRI.

If you require further information please contact Professor Marsh or any other member of the research team at the KEMRI centre, Kilifi. (Telephone 0125 22063)

If you want to ask someone independent anything about this research please contact
Dorcas Kumuya at KEMRI – Kilifi, Tel: 041 522 063

Or
Mr. Ambrose Rachier, Chairman - KEMRI/National Ethics Review Committee
P. O. BOX 54840-00200, Nairobi, Tel number: 020 272 2541 Mobile: 0722205901 or 0733400003

THE PARENT/GUARDIAN SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

OXTREC NO: 030-06 240

Consent form 3
Integrated studies of the development of natural immunity to malaria in children in Kilifi district

Lead Investigator: Kevin Marsh, Director KEMRI-Wellcome Programme.

When you sign below it shows that you have read the information about the study and have been able to have any questions answered and that you have agreed to join the study. If you do not understand any part of the information sheet ask questions. Participation in the study is voluntary and you have the right to change your mind and withdraw from the study at any time without giving a reason. Do not sign until you have answers to your questions.

I agree to take part in the study of how children develop immunity to malaria. I understand that I will be asked permission to take a 5ml blood sample from my child during this illness and that I will be asked to return to clinic in approximately three weeks time for a second blood sample to be taken. I understand that some of the blood samples may be exported to laboratories outside Kenya. ☐

THIS SECTION TO BE READ ONLY FOR THE SUBSET OF CHILDREN INVITED TO PARTICIPATE IN EXTENDED FOLLOW UP:

I understand that I will then be asked permission to return to clinic on three more occasions at approximately six, twelve and twenty four weeks from now ☐

I agree to blood being stored for testing in the future. ☐
(tick boxes to indicate consent, place a cross in the box to indicate lack of consent)

Childs name_____

Signature (or thumb print) of parent_____

Name in capitals _____

Date _____

I have read the information sheet for "Integrated studies of the development of natural immunity to malaria in children in Kilifi district" to_____ (parent"s name) in a language they understands. I believe they give consent for_____ (child"s name) to take part in the study.
Signature of translator_____

Name in capitals_____

Date_____

Only necessary if the parent/guardian cannot read:

I *attest that the information concerning this research was accurately explained to and apparently understood by the parent/guardian and that informed consent was freely given by the parent/guardian.

Witness' signature: _____ **Date** _____

Witness' name: _____ **Time** _____
(Please print name)

***A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.**

OXTREC NO: 030-06 241

APPENDIX 2.1B
Information sheet (Children in cohort studies)
Integrated studies of the development of natural immunity to malaria in children in Kilifi district

Lead Investigator: Kevin Marsh, Director KEMRI-Wellcome Programme.

What question is KEMRI trying to answer?

KEMRI is an institution which learns more about diseases to find better ways of preventing and curing those diseases. An example is malaria, which causes much death and illness among Kenyan children. Children are protected against measles by the measles vaccine. Children could be protected against malaria if there was a malaria vaccine. In areas like this people become immune to the bad effects of malaria as they grow older. In KEMRI we are working to try to understand how people become immune to malaria. This information is important in designing vaccines against malaria.

What does taking part involve?

We want to find out why some people get more episodes of malaria and other illnesses than others. In order to do this we need to follow group of children over time to see how often they have malaria and other diseases. We also need to study how some of the components of the child"s blood that fight diseases change over time. We need to follow about 500 children in this community. We plan to follow them over the next four years.

We are requesting your permission for your child to be one of these children. If you agree, the study will involve:

1) **Weekly visits to your home.** Fieldworkers living in this community will visit you and your child each week to ask if your child is well. At each visit we will take your child"s temperature to see if they have a fever. If they are unwell but don"t have a fever the fieldworker will return twice over the next 24 hours to recheck the temperature. If the child does have a fever they will collect a finger prick blood sample. Some of this blood will be tested immediately to see if the child has malaria. If your child does have malaria the field worker will report this by telephone to a KEMRI doctor and we will provide treatment for your child. The field worker will call someone from the dispensary to take a 5 ml blood sample from your child. The blood will be taken to the KEMRI laboratories for other tests which are described below. **The fieldworkers will only be able to feed back malaria results, and they are not doctors.** If your child has another sort of problem, the fieldworker will advise you to visit the dispensary where KEMRI staff are working with the Ministry of Health staff to diagnose and treat illness.

2) **Four additional blood samples at set times in the first year, even if your child is well.** On three of these occasions the blood sample will be collected by finger prick. On one occasion a sample of 5mls (one teaspoon full) will be collected from a vein on your child"s hand or arm.

3) **After the first year one blood sample of 5 mls once a year for the next three years unless your child has malaria when an additional blood sample will be taken.**

Are there any risks to being in the study?
Taking a blood sample by finger prick or by needle can be uncomfortable and can sometimes cause mild bruising. The amount of blood taken is completely safe and children are able to replace blood themselves.

Are there any benefits to being in the study?
By having a weekly visit by the KEMRI fieldworker your child will have a regular check to see if they are unwell. If they have malaria they will receive immediate treatment and if they have other illnesses they will be referred to the local dispensary to be seen by a nurse of clinical officer working with KEMRI In order to make sure that children with malaria or other illnesses have good access to care on days when the field worker is not visiting, KEMRI have placed extra nursing and clinical officer staff at the local dispensary. This benefits people in this community whether they are in the study or not in the study.

What happens to the blood samples taken in the study?

We want to find out why some people get more episodes of malaria and other illnesses than others. In the laboratory we will examine the blood to see if it contains substances or cells which recognize and protect against the malaria parasite. Some of these substances are "genetic" which means they are present from birth and some are things that develop as we grow older after we have been exposed to malaria.
Most of the tests will be done in the laboratories in Kilifi. Some of the tests cannot at present be done in Kenya and so will be done in laboratories overseas. Not all the tests will be done at the same time and so the blood will be stored in a freezer until the tests can be done. At the end of the study any blood that has not been used in tests will be stored so that further tests of the kind described above can be carried out in future.

(To be read to parents of children who are already in the cohort recruited under previous SCC protocol:
Your child has already been in this study for some time and we are now inviting you to continue in the study. We will ask you separately at the end of this form for permission to use continue to use samples already taken from your child up until this time and stored in Kilifi.

How will my rights be protected?

This research has been reviewed by a group scientists and doctors in Nairobi to make sure that the research is well designed. It was then reviewed by the Kenya national ethical committee. This is a committee of people from different walks of life, including lawyers and religious leaders, whose job is to make sure that the research conforms with national and international standards for safety and for protecting the rights of people who take part in the research. Any changes to the study that we want to make have to be approved first by the ethical committee. No tests or procedures other than the ones you have agreed to can be carried out. (Out of curiosity- is there a way in Swahili/Giriama to describe ethical?)

What if I decide not to involve my child or change my mind later?

Participation in research is completely voluntary. Nobody is obliged to take part and anyone who does agree can change their minds at any time. If you refuse to take part, or agree and change your mind later, there are no penalties what so ever, and you and your child will still be able to benefit from the improved level of service offered at the dispensary and at Kilifi hospital as a result of the research carried out by KEMRI.

If you require further information please contact Professor Marsh or any other member of the research team at the KEMRI centre, Kilifi. (Telephone 0125 22063). You may also contact the National Ethical review Committee on 02 2722541

OXTREC NO: 030-06243

Consent form

Integrated studies of the development of natural immunity to malaria in children in Kilifi district

Lead Investigator: Kevin Marsh, Director KEMRI-Wellcome Programme.

When you sign below it shows that you have read the information about the study and have been able to have any questions answered and that you have agreed to join the study. If you do not understand any part of the information sheet please ask questions. Participation in the study is voluntary and you have the right to change your mind and withdraw from the study at any time without giving a reason. Do not sign until you have answers to your questions.

I wish my child to take part in the study of how children develop immunity to malaria. I understand that this will involve weekly visits to my house by a KEMRI fieldworker for a period of up to four years. I understand that in the first year I will be asked permission to take a blood sample from my child on four occasions and that in subsequent years I will be asked permission to do this once a year or when my child has malaria. I understand that some of the blood samples may be exported to laboratories outside Kenya ☐

I consent to my child"s blood being stored for testing in the future.
(Tick boxes to indicate consent, place a cross in the box to indicate lack of consent) ☐

I agree to blood being exported to laboratories outside Kenya for further tests.
(Tick boxes to indicate consent, place a cross in the box to indicate lack of consent) ☐

Childs name_____

Signature (or thumb print) of parent_____

Name in capitals _____

Date _____

I have read the information sheet for "Integrated studies of the development of natural immunity to malaria in children in Kilifi district" to_____ (parent"s name) in a language they understand. I confirm that they give consent for_____ (child"s name) to take part in the study.

Signature of translator_____

Name in capitals_____

Date_____

If you require further information please contact Professor Marsh at the KEMRI centre, Kilifi. (Telephone 0415 22063) You may also contact the National Ethical review Committee on 02 2722541

OXTREC NO: 030-06

8.2: Working protocol for combined IFA/FACs

FACS/IFA with assorted anti-NTS DBLalpha antibodies at 400 μg/ml
date: / /11

Aim: A combination of FACS and IFA to test whether the anti-NTS DBLalpha antibodies cross-react with different rosetting strains at high concentration parasite smear

Need 1.2 ml of culture suspension (mature pigmented trophs, preferably NOT schizonts) at 2% haematocrit. Make a 13.5μl working stock of Hoescht by adding 1μl of the stock to 12.5μl of PBS. Add 2μl of working stock to 1.2 ml of culture to get a final concentration of 1.25μg/ml. Incubate at 37 degrees for 20min. Spin down and wash 2x in PBS. Resuspend in 1.2 ml PBS/1% Ig-free BSA/1.25μg/ml Hoescht (solution 3 on next page). Need 11x 94.6μl aliquots.
Add primary Ab as below to give final conc of 400 μg/ml of antibody. All Abs are at 7.5 mg/ml (cold cabinet, right hand side, 2nd shelf, red tray, Alex antibodies box. Red lids).

Samples:
1. No Ab Ctl add 5.4 μl of PBS
2. Rabbit IgG control add 5.4 μl of Ab
3. anti-R29 DBLalpha rabbit 5776 add 5.4 μl of Ab
4. anti-TM180 DBLalpha rabbit 6221 add 5.4 μl of Ab
5. anti-TM284 DBLalpha rabbit 6219 add 5.4 μl of Ab
6. anti-PAR+ DBLalpha rabbit 6217 add 5.4 μl of Ab
7. anti-HB3var6 DBLalpha rabbit 6223 add 5.4 μl of Ab
8. anti-Muz12 DBLalpha rabbit 6283 add 5.4 μl of Ab
9. anti-HB3var3 DBLalpha rabbit 6358 add 5.4 μl of Ab
10. mouse anti-human IgM (Serotec) add 0.5μl Ab and 4.9□l of PBS
11. No Ab Ctl add 5.4 μl of PBS

Incubate on ice for 1 hour, resuspend samples every 10 mins as usual. Wash 3x in cold PBS (750μl per wash). Spin secondary Abs for 2 mins in microfuge 13000 rpm before use below.

Make 1 ml of secondary Ab Alexa488 goat anti-rabbit IgG at 1/1000 in PBS/1% Ig-free BSA 1.25μg/ml Hoescht (solution 3). Add 100μl to pellets in tubes 1-9. You will need to make a different secondary reagent for tubes 10 and 11. Make 300μl of secondary Ab Alexa488 goat anti-mouse IgG at 1/500 in PBS/% Ig-free BSA,/1.25μg/ml Hoescht. Add 100μl to pellets in tubes 10 and 11.

Incubate on ice for 1 hour (protect from light), resuspend samples every 10 mins as usual.

Wash 3x in cold PBS (750 μl) as usual. At last wash, split into two sets of tubes 1-11 (for IFA) and 1*-11* (for FACS).

IFA: continue as usual with tubes 1-11, by resuspending at 20-30% haematocrit in PBS/1% Ig-free BSA, make smears, air dry, add DABCO/glycerol mountant and coverslip, and store at 4°C.

FACS: Remove supernatant from tubes 1*-11* and resuspend pellet in 100µl of PBS/1% Ig-free BSA/50µg/ml fucoidan. Incubate for 5 min and check that the rosettes have been disrupted (record). Spin down and resuspend in 200µl of 0.5 % paraformaldehyde and incubate on ice for 20 min. Wash once in cold PBS and once in FACS buffer (750 µl) as usual. Resuspend pellet in 500µl of FACS buffer and transfer into a FACS tube, add 25µl of 1mg/ml fucoidan and incubate for 20 min at RT. Foil wrap the tubes and keep in the fridge till when you do the FACS run (preferably within two days of staining).

NB-Summary of working stocks required for this assay (Make 1-3 before starting the assay to make life easy):
For initial staining and subsequent use
1) Hoescht (13.5µl)-
1µl of stock (10mg/ml brown bottle)
12.5µl of PBS

2) 5ml of PBS/1% Ig-free BSA
167µl of IgG-free BSA
4.83 ml of PBS

For primary and secondary incubations
3) PBS/1%BSA/1.25µg/ml Hoescht/ (3 ml)-
3 ml of above PBS/1% Ig-free BSA
5µl of above Hoescht

For rosette disruption step
4) PBS/BSA/Fucoidan (1.5 ml)-
1.43 ml of above PBS/1% Ig-free BSA
75µl of 1 mg/ml fucoidan

8.3 Working protocol for Phagocytosis assay

Coat 96-well round-bottom plates with 200µL/well of neat FBS (can be re-used for coating several times) for 1h at RT.

Wash wells with 200µl sterile PBS (3x), let air dry inverted on a piece of paper towel and store at 4 degrees. (use within a month). This is all done in the hood.

To the opsonisation plates add the desired amount of antibody for a final volume of opsonisation of 33µL. (e.g. for a 1/10 dilution use 3.3µL of antibody) Freeze the plate with antibodies until parasites are ready to be used. The antibody samples are diluted down from a working stock of 4mg/ml which is made from the original supply stocks. Using the working stocks at 4mg/ml , each antibody sample is then diluted 4-fold to 400µg , 100µg , 25µg and 6.25µg/ml.

OPTIONAL: coat 15ml and 50ml tubes with neat FBS (these can be used to collect the parasites and wash them. The FBS coating prevents the adherence of pRBC to the sides walls of the tubes, thus minimizing losses)

Culture your Thp-1 so that they will be at between 400,000 and 700,000 cells/mL on the day of the assay (usually if they are at 350,000 cells/mL on the morning before the assay, that results in perfect 500,000 cells/ml on the day of the assay).

Cell lines used:
Any parasite culture as target of antibodies
Undifferentiated THP-1 cells

Solutions needed:
Stock solution of 20mg/ml of Fucoidan
Incomplete parasite medium
Incomplete parasite medium + 200ug/ml Fucoidan + 2% FBS or 0.5% BSA (RPMI-F)
FACS Buffer (PBS minus Calcium and Magnesium, 2% FBS, 2mM EDTA, 0.02% Azide). Addition of azide is optional.
THP-1 complete media (RPMI1640, 10% HI FBS, glutamine, Pen-Strep, 25mM HEPES, 55µM 2 ß-mercaptoethanol). The 2ME is added to culture flask when changing the media at a 1:1000 ratio. 5ml each of glutamine and Pen-Strep as received from stores stocklist – check actual concentration later and write in.
THP-1 complete media + 200µg/ml Fucoidan (THP-F) – used in Phago-assay.
FACS Lysing Solution 1/10 in ddH2O
2% PFA (formaldehyde can be used) in PBS minus Calcium and Magnesium. This can be made up using 37% Formalin (1ml of Formalin diluted in 18ml of PBS-).

Methods:

1 – Parasite isolation by MACS (using Fucoidan)
2 – Phagocytosis assay in the presence of Fucoidan

1- Parasite isolation by MACS (using Fucoidan)

Method for heparin-insensitive rosettes In this modification of the standard MACS method (Staalsoe et al, Cytometry 1999), rosettes are disrupted with fucoidan before and during purification to allow collection of rosetting PRBC free from uninfected red cells.

To prepare the column
Fit the column with the 3 way tap, 20G needle (yellow) and syringe as per manufacturers instructions. Cut the end off the plastic needle protector, while keeping the sharp end of needle covered. Fit the column into the magnet. Add 30ml RPMI-F to a 50mL syringe and assemble with the 3-way tap.
Through the lower 50ml syringe force 10 ml of media up and down the column to dislodge any air bubbles trapped inside it.
Open the 3-way tap and allow the medium to run through at approx 1 drop/sec
Then run through at same speed approx 10-20 ml incomplete RPMI followed by 5ml incomplete RPMI 2% serum with 200 μg/ml Fucoidan (add solutions to top of reservoir).

The column is now equilibrated and ready to use. Switch tap to "off" position.

To prepare the parasites
% parasitaemia:_____ (yields from cultures less than 5% trophs will be poor)
Rosetting Frequency:_____

-Take parasite culture and spin down.
-Wash 1x in incomplete RPMI. Pipette up and down vigorously (helps disrupt the rosettes)Spin down, remove supernatant and resuspend the cells in 1mL of RPMI-F for every 2ml of original parasite culture (if original culture at 2% hematocrit).
-Incubate 5-10 min at RT. Check a wet prep under microscope (40x objective) to ensure all rosettes disrupted.
-Add parasite suspension to column and run through at approx 1 drop/sec.
-Collect flow through and run through the column a second time.
-Collect final flow through put a drop on a slide, cover with a coverslip and check for the presence of trophs under light microscopy. Check that PRBC have been removed.
-Wash column with 10-20ml of RPMI-F until flow through is completely clear. Add a further and final 5ml wash.
-Remove column from magnet keeping the 50ml syringe attached.
-Force the remaining 20ml of RPMI-F left in the syringe up and down(5-10 times) the column to elute the adherent PRBC. Pull everything into the syringe
-Dispense the eluate into a 50ml Falcon and smear or look at wet prep to check purity. Should get a good yield (usually 50-80% of "theoretical" yield) and a parasitaemia of 80-100%.
-Wash the column with H20, then flow 100% methanol through it and store when dry or leave it in 100% methanol at 4 degrees.

2. Phago assay in the presence of 200 μg/ml of Fucoidan.

-After the isolation of the parasites has taken place resuspend parasites in 1 ml of RPMI-F.

-Count parasites on haemocytometer (put 5µl of parasite into 1ml of PBS: 200x dilution). Haemocytometer formula: Parasite count in each of 4x4 squares/4 x dilution factor (200) x 10,000 = parasites/ml
-Resuspend the parasites in an FBS-coated tube at 3.3x10^7/ml in RPMI-F + 10 µg/ml of EtBr (100x dilution) (wrap in aluminium foil and work as much as possible in the dark from now on whenever dealing with parasites). BE AWARE THAT ETBR IS CARCINOGENIC.
Wait for 15 min (optionally with gentle agitation)
-Wash at least 3x with RPMI-F (transferring parasites to at least one new tube during the washes) – IF YOU HAVE A FROZEN PLATE WITH ANTIBODIES TAKE IT OUT OF THE FREEZER NOW
Resuspend again at 3.3x10^7/ml in RPMI-F.
Transfer 30µl to a 96-well plate into all wells that contain antibody amounts as planned and also into your control wells.

Your wells should include:
Negative control: Control Rabbit (neg)
Negative control: Un-opsonised (no antibody)
Common Positive control: Rabbit anti-human RBC from ABCAM @90 µg/ml and if using plates to be compared on different days 8 dilutions of 2x or 4x (start at higher concentrations when using multiple plates of human serum).

NOTE: for Palo Alto the common positive control is always lower than for HB3, TM180R+,TM284R+, R29R+... that is a reflection of the binding of the rabbit antibody to the surface of the PAR pRBC.

Samples
THP-1 alone

-Mix well and leave for 1h at RT covered in foil.
-15min before the hour finishes get your Thp-1 cells, count them and resuspend at 5x10^5 cells/ml in Thp1-F media. You will need to work out how many duplicate wells will be required and then factor up accordingly the total volume of cells needed.
-Add 150ul of RPMI-F to your parasites and spin at 300G for 4 min at RT (this is program 1 on the centrifuge). Discard supernatant (by quickly inverting the plate and blotting on paper towels) – note risk of EtBr on bench and sink.
-Repeat this 150ul wash and spin step 3 times.
-Resuspend parasites in 100ul of THP-F media.
-Transfer 100µl of Thp-1 cells in DUPLICATE and 50ul of parasite into each well of a new 96-well plate. Mix well and take to the 37 °C CO_2 incubator on the ground level culture Lab. Allow 40 min for the phagocytosis to occur. (During this 40 minute incubation, run program 2 on plate centrifuge to lower temperature to 4 degrees. Put FACS Lysing Solution in 37 degrees water bath.)
-After 40 minutes put the plate on ice.Spin down plate at 4 degrees 300G for 4 min (program 2). Discard supernatant by inversion and blotting onto tissues.
Resuspend in 150µl of warm FACS Lysing Soln. Mix well. Wait for 10 min.
Add 50ul of ice-cold FACS buffer to each well.
-Spin down plate at 4 degrees 300G for 4 min (program 2). Discard supernatant by inversion and blot onto tissues.
-Add 150ul of ice-cold FACS buffer to each well , and repeat step18.
-Resuspend each well in 150µl of ice-cold PFA 2% in PBS minus calcium and magnesium.

-Allow for 2h at 4 degrees before taking to the FACS machine to read. You can leave overnight covered in foil and at 4*C without any problems.

Experimental Notes and results:

Parasites Before Isolation:
Parasitaemia:
%RF:

After Isolation:

___x 200 (dilution factor) x 10,000 = ___ml x 0.7 (parasite loss due to washes from now on):

Final parasite: _____
Resuspend in : _____
Thp-1:

____ x (dilution factor) x 10,000 = _____ml:
resuspend in : _____

FACS Protocol for acquisition Phagocytosis experiments – Current rough notes
FACS bookings online page:
http://www.iiirflow.biology.ed.ac.uk/iiir_flow_index.html

Just click on "booking diary" and then select a day and hour. You can use my username and password (both are ricardoataide) to book or alternatively ask Martin to give you a username and password.

-If need be, restart FACS computer.
-Switch ON FACS machine. Flick Black Toggle switch DOWN in reservoir drawer.
-Facs machine Mode is RUN and HI
-Click on Peoples Folders, select Ahmed, select Template Folder , select THP-1 .
-Select ACQUIRE clickmenu link, select Connect to Cytometer.
-Click Apple Icon and select 1, 2, 3, 4
-Select Instrument Settings clickmenu link, select Open and double-click on file you want. Then click on SET, and then click on DONE
-DIRECTORY setting is Ahmed -> New Folder -> Expt. Name with Date info as : YY/MM/DD Then click Create , then click Choose , Click File -> Sample ID
-At this point, make ready the FACS machine to read samples. When cleaning or priming FACS machine, the Set-Up box MUST be ticked. When ready to read samples at any point then Set-Up box is to be unticked.
FACS machine is Primed by flick of lever to the right, and then quickly placing a FACS tube of PBS into the Sample Feed Slot , making sure it is firmly in to the top of the slot.
-Flick lever back to the centre position, click Acquire , run for a few seconds , click -Pause and then click Abort.
-At this stage , you can check your control samples to make sure all is well with the assay.
-Acquire click menu link ; Counters ; G ; Accept -> Collect
Source : Mode :
Collect : G1 Rate :

Collect : G1
-When ready to read samples , untick the Set-Up Box , type in sample ID info , click Acquire and let sample run for selected Event Counts or click Pause and Save if counts are low or if sample is about to run out.

-After all samples have been read , data is automatically saved into the file so no need to worry about saving anything , the FACS machine needs to be cleaned , rinsed , emptied of waste and refilled with FACS buffer. Tick the Set-Up Box , then run 2ml of Clean fluid with the lever set to the right , then run 2ml of Rinse fluid , then run dH_2O for 1 minute and at same time , click Acquire to check that 0 events are detected. Then click Pause and Abort. Empty Waste Tank and Refill FACS Buffer Tank.

-Acquire clickmenu link -> Disconnect , then CellProQuest -> Switch Off
Flick Black Toggle Switch Back Up , Press StandBy Button and Low Button
Switch Off FACS Machine

8.4: FASTA sequences from rosette selected field isolates

1. SA075 cDNA and gDNA sequences from R+ and R- clones

Rosette positive clone (R+) cDNA sequences
>R+_11_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_14_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_10_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_13_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_16_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_4_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_8_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_1_cDNA

DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R+_9_cDNA
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRDQVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLDYVPQF
LR
>R+_2_cDNA
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRDQVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLXYVPQF
LR
>R+_3_cDNA
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRDQVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLXYVPQF
LR
>R+_12_cDNA
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRDQVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLDYVPQF
LR
>R+_15_cDNA
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRDQVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNVPTNLXYVPQF
LR
>R+ 16 cDNA
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRDQVWKAITCKAPEEDHYFKPAQNRKREFTDGHCGHRQGNXPTNLXYVPQF
LR
>R+_5_cDNA
DIGDIIRGRDLYRRDKGGKTKLEKNLEKIFQYIKENNKSSLESLTDEELREDWWAL
NRDQVWKAITCHAEHSDEYFRKSRDGVYLYFDDRCGRDLSSVPTNLDYVPQFL
R
>R+_6_cDNA
DIGDIVRGKDLYLGTDKDDVEKGLKIVFQKINNGLKKIGINAYDDGSGNYYKLREV
WWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLDY
VPQFLR

Rosette negative clone (R-) cDNA sequences
>R-_9_cDNA
DIGDIIRGKDLYIRNKREKRRLEDNLQKIFKELKRGVKRKEAEKHYEDGAPEFYKL
REDWWDANRLEVWKAITCEAQGFNYFRRTCSGGTSTTHGKCHCIGGTVPTYFD
YVPQYLR
>R-_2_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_3_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_16_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR

>R-_12_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_7_CDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_1_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_4_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_5_cDNA
DIGDIVRGRDMFLPNKDDKVQKGLQVVFEKINNGLKKIGINAYNDGSGNYSKLRE
VWWNVNRDQVWRAITCSAPGDVNYFRKISGDTRTFENAGKCRRHDNKVPTNLD
YVPQFLR
>R-_14_cDNA
DIGDIIRGKDLFRGNNKEKKQREKLDENLKTIFANIYKDVTSSGRNVQKLQKRYND
DNENYYQLREDWWTANRETVWKAITCSEKLSNSKYFRGTCGGDENTATRTPSH
CRCDGKNADQVPTYFDYVPQYLR
>R-_6 cDNA
DIGDIIRGKDLYLGNKRQNETEREKKKLQQNLKEIFKKIYEGLKGDAQKDYKDESG
NYYQLREDWWNANRQEIWKAMICKAPGNAQYFRNTCSNGEKPTDEKCQCIDGT
VPTNLDYVPQFLR
>R-_8 cDNA
DIGDIIRGRDLYGGNKKKERLEDNLKKIFRNIYDKLLEENRTNGELKTRYQDDGSG
NYYQLREDWWTLNRQDVWKAITCDDRLGGYSYFRATCGDSESPSMARDKCRC
KKKNGRPDDQVPTYFDYVPQYLR
>R-_10 cDNA
DIGDIIRGKDLFLGYNERDRAQKKKLQDNLEKIFGKIYKDLTNGAQNHYKDENGG
NFFKLREDWWDAXRLEVWKAITCDAGQNDKYFXQTCDDNGTSSNANHKCRCK
KNDDTSDTDQVPTYFDYVPQYLR
>R-_11 cDNA
DIGDIVRGRDLYRGNNGKDKLQDNLKKIFXXXHDKLDNSIKSKYNDDTENYYKLR
EDWWTANRETVWKAITCEAYGTYFRATCNSGDNRGCSQAKDNCRCKDEEGKX
XXDQVPTYFDYVPQYLR
>R-_15_cDNA
DIGDIIRGKDLYIRNKREKRRLEDNLQKIFKEIYEELKRGVKRKEAEKHYEDGAPEF
YKLREDWWDANRLEVWKAITCEAQGFNYFRRTCSGGTSTTHGKCHCIGGTVPT
YFDYVPQYLR

Rosette positive clone (R+) gDNA sequences

>R+_9_gDNA
DIGDIIRGKDLFLGYNERDRAQKKKLQDNLEKIFGKIYKDLTNGAQNHYKDENGG
NFFKLREDWWDANRLEVWKAITCDAGQNDKYFRQTCDDNGTSSNANHKCRCK
KNDDTSDTDQVPTYFDYVPQYLR
>R+_2_gDNA

DIGDIIRGKDLYRGNSKEKDNLEKKLIEYFKELHKHLDQKTKNHYTKDDPYYYKLR
EDWWNANRKEAWKAITCKAENAQYFRNTCSMGKHSTQTNCQCIDGEVPTYFDY
VPQYLR
>R+_10_gDNA
DIGDIIRGKDLFLGTTQEKKSLEENLKNIFRKLYNELTKEEENGTAIKSRYENDGPN
YYQLREDWWALNRKEIWKAITCDTEESDTYFKQSSEGKYSFTNGQCGHNEENV
LTNLDYVPQFLR
>R+_3_gDNA
DIGDIVRGKDLYRGDKGEKRRKKKLEERLKTIFGKIHEEVTNGSNGQTLQARYKD
DKENFFKLREDWWYANRGTVWKAITCEAPESAQYFRNTCNGGEQTKGYCRCN
DDKPDDDKANVDPPTYFDYVPQFLR
>R+_4_gDNA
DIGDIVRGKDLFLGNTYESARRDKLEQKLKDIFKEIHGGLKGAKTRYNGDGDNYF
QLREDWWNANRETVWKAITCGADAGNKYFRPTCGGHNENTTFRTPSQCRCSD
NQVPTYFDYVPQYLR
>R+_5_gDNA
DIGDIVRGRDMFKRTDKDEVKEGLQVVFKKIYNTLPSPAQNYYADESENYYKLRD
AWWTANRDQVWEAITCKAPQKVDYFRKGSNGESIFSNSGKCGRNETDVPTNLD
YVPQFLR
>R+_13gDNA
DIGDIIRGKDLYRGDSKEKDNLEKKLIEYFKELHKHLDQKTKNHYTKDDPYYYKLR
EDWWNANRKEAWKAITCKAENAQYFRNTCSMGKHSTQTNCQCIDGEVPTYFDY
VPQYLR
>R+_6_gDNA
DIGDIVRGKDLYLGDKKEKKQLEENLKTIFGNIYKELTTENGVKARYKDTDNYYEL
REDWWNANRNDIWKALTCDAPKDAQYTKKGPHNHITESNRGQCRCFSGDPPT
NMDYVPQYLR
>R+_14_gDNA
DIGDIVRGKDPFYGNTQEKEKREDLENNLKTIFGKIHNGLHDKIKSKYNDDAKKNY
YQLREDWWYANRQEVWKALTCDVDGSYFHATCNGEERTKGYCRCDGDKSKV
GTNETDQVPTYFDYVPQFLR
>R+_15_gDNA
DIGDIVRGKDPFYGNTQEKEKREDLENNLKTIFGKIHNGLHDKIKSKYNDDAKKNY
YQLREDWWYANRQEVWKALTCDVDGSYFHATCNGEERTKGYCRCDGDKSKV
GTNETDQVPTYFDYVPQYLR
>R+_8_gDNA
DIGDIVRGKDLFRGNNKEKKQREKLDENLKTIFANIYKDVTSSGRNVQKLQKRYN
DDNENYYQLREDWWTANRETVWKAITCSEKLSNSKYFRGTCGGDENTATRTPS
HCRCDGKNADQVPTYFDYVPQFLR
>R+_16_gDNA
DIGDIIRGKDLFRGYNEKDQEEKTKLQENLKNIFKKIHENLGDTTIKSKYNDDTDKN
YYKLREDWWDLNRDQVWKAITCEAPGDAKYKVIGSDGSTTESARGQCRGVADV
PTNFDYVPQYLR
>R+_11_gDNA
DIGDIIRGKDLFLGYNERDRAQKKNYKTIWKKFSGKYIRISTKKKLQDNLEKIFGKIY
KDLTNGAQNHYKDENGGNFFKLXEDWWDANRLEVWKAITCYAGQNDTYSINTG
NSITEFWFGRCGRNERNVPTNLDYVPQYLR

Rosette negative clone (R-) gDNA sequences
>R-_9_gDNA
DIGDIIRGKDLYLGDNGKDRLENKLKEIFKQIYDKLDGKKKKEAKARYQDDGSGN
YYQLREDWWNNNRKMVWYAITCGAENDSTYFRNTCGSGEKGSATTGKCRCPK
ANANQVPTYFDYVPQYLR

>R-_10_gDNA
DIGDVVRGKDLFLGNTYESARRDKLEQKLKDIFKEIHGGLKGAKTRYNGDGDNYF
QLREDWWNANRETVWKAITCGADAGNKYFRPTCGGHNENTTFRTPSQCRCSD
NQVPTYFDYVPQYLR
>R-_3_gDNA
DIGDIVRGRDLYRRDKGGKTKLEKNLEKIFQYIKENNKSSLESLTDEEIREYWWAL
NRDQVWKAITCHAEHSDEYFRKSRDGVYLYFDDRCGRDLSSVPTNLDYVPQFL
R
>R-_11_gDNA
DIGDIIRGKDLFLGTTQEKKSLEENLKNIFRKLYNELTKEEENGTAIKSRYENDGPN
YYQLREDWWALNRKEIWKAITCDTEESDTYFKQSSEGKYSFTNGQCGHNEENV
LTNLDYVPQFLR
>R-_12_gDNA
DIGDIVGRDLYSGNDEEKKQRKQLDKKLKDIFKNIKNENKDLTTLKPEEVREYWW
DANRATIWYAITCGAGQSDKYFRATCGGGKTPTQGKCRCSDNPNTDPPTYFDY
VPQYLR
>R-_16_gDNA
DIGDIVRGRDLYSGNDEEKKQRKQLDKKLKDIFKNIKNENKDLTTLKPEEVREYW
WDANRATIWYAITCGAGQSDKYFRATCGGGKTPTQGKCRCSDNPNTDPPTYFD
YVPQYLR
>R-_5_gDNA
DIGDIIRGKDLYLRYNRKDKTDKLQEQLKKYFQKIYDQLKSQAKEYYKEDKRDGN
FYQLREDWWTANRAKVWDAITCKAGSDSQYFRRTCGSGNNAKRTPSQCRCTT
RVVPTYFDYVPQYLR
>R-_13_gDNA
DIGDIIRGKDLFYGNKKEKNRREKLEDNLKRXFGHIYEELKKDRKKSALAKERYGS
DTTNYFQLREDWWALNRDQVWKAMTCSEHLKNSAYFRPTCSDSHRSGTFSQA
KDKCRCKDEKGKNETDQVPTYFDYVPQYLR
>R-_7_gDNA
DIGDIIRGKDLYRGNRKKNQNETEREKLEQKLKEIFAKIYNSLKNKEDAKKHYGGD
APYYYQLREDWWTANRAKVWEAITCHVVSGNNYFRHTCGARKDRTQTQDNCQ
CIDQTVPTYFDYVPQYLR
>R-_1_gDNA
DIGDIVRGRDLYLGDKKEKDRLQSTLKRIFRKIYKNLMEDLTKSNRTKVAEAQTRY
NGDDNYYQLREDWWEANRQEIWKAITCEHPGGTYFRQRACAGRFSTDDKCRC
VTDVPTYFDYVPQYLR

2. cDNA and gDNA sequences for the rosette selected samples

9106R+ cDNA sequences

>9106R+7
DIGDIIRGKDLFIGYDQKEKDRRENLENKLKKIFEKIHKEVTSTSGRRNRQTLKARY
DGDTTNYYQLREDWWNANRIMVWIAMTCGAGESDKYFRKACSGGTTPTNKKC
RCTTHDVPTYFDYVPQYLR
>9106R-3
DIGDIIRGKDLFIGYDQKEKDRRENLENKLKKIFEKIHKEVTSTSGRRNRQTLKARY
DGDTTNYYQLREDWWNANRIMVWIAMTCGAGESDKYFXKACSGGTTPTNKKC
RCTTHDVPTYFDYVPQYLR
>9106R+9

DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR
>9106R+2
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR
>9106R+8
DIGDIIRGKDLYRGNKQEKEKREQLENKLKIYFTQIHENLGTQEKNHYNDTKNYFQ
LREDWWTANRATVWKAITCRAGEGDRYFRATCGSGRDATRARDKCRCEGANV
VPTYFDYVPQYLR
>9106R+10
DIGDIVRGRDLYLGNPQEKTKREQLEKNLKDIFKEIHDDVMKTSGRNGKKVEAEK
RYNDTTNYFQLREDWWEENREKVWKAITCNAGGGKYFRQTVCSEGTTPTNEK
CQCINFSVPTNFDYVPQYLR
>9106R+11
DIGDIIRGKDMFKRTDKDDVENGLREVFGKEYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR
>9106R+4
DIGDIIRGKDMFKRTDKDDVENGLREVFGKEYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR
>9106R+12
DIGDIIRGKDMFLGNNKEKKQREQLDDKLKEVFGKIHGGLIKNGRNGEAAQEYYE
DKGKNYYKLREDWWTANRATIWEALTCHAPPDAQYFHATCGTGTGTQGRCRC
GDGKSKVGKNETDQVPTYFDYVPQYLR
>9106R+5
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR
>9106R+6
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR
>9106R+1
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR

9106R- cDNA sequences
>9106R_9
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR
>9106R-2
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQSLR
>9106R-10
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR

>9106R-11
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR
>9106R-4
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR
>9106R-12
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR
>9106R-6
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
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YVPQFLR
>9106R-1
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR
>9106R-7
DIGDIIRGKDMFKRTDKDDVENGLREVFGKIYKSLNGKAISHYTDTHGSIDYVKLR
EDWWTANRDQVWRAITCYIPYYVNYFKKTSDDIIVFTNDGKCGHYEGAPPTNLD
YVPQFLR

9106R+ gDNA sequences

>9106R+2gDNA
DIGDIVRGRDMFKPNSDDKVQEGLKVVFRKINDGLKKSRINDYDRDGPEYYKLRE
DWWTANRNQVWRAITCAARYNPGYFKKSDRSTQVFSNPKCGHDEGIVPTNLDY
VPQFLR
>9106R+10gDNA
DIGDIVRGKDLYIRNKKKDKLEENLKNMFQYIKTKHNTKLGQLTLDQVREYWWAL
NRKEVWKAMTCSDDNKLANASYFRQTCDDNGTSSNANHKCRCKDKKGEHDTD
QVPTYFDYVPQYLR
>9106R+3gDNA
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DNENYYQLREDWWTANRETVWKAITCSEKLSNSKYFRGTCGGDENTATRTPSH
CRCDGKNADQVPTYFDYVPQFLR
>9106R+11gDNA
DIGDIIRGKDLYLRYNRKDKTDKLQEQLKKYFQKIYKELTWTATKNGQKSAKDHY
KDDTGDFFQLREDWWNNNRKMVWYAMTCGAGTSAQYFRKTCGSGNWTKDKC
RCTTHDVPTNFDYVPQYLR
>9106R+4gDNA
DIGDIIRGKDLFIGYDQKEKDRRENLENKLKKIFEKIHKEVTSTSGRRNRQTLKARY
DGDTTNYYQLREDWWNANRIMVWIAMTCGAGESDKYFRKACSGGTTPTNKKC
RCTTHDVPTYFDYVPQYLR
>9106R+13gDNA

DIGDIIRGRDLYLGNKKKSENKREKEKLERKLKSFFKNIYDDLINDQGKKDAAIKHY
FDPKGNYYQLREDWWDLNRHDVWKAITCSAPNEAKYNVIASDGTTTKSNYKKC
RNIADVTTYFDYVPQYLR
>9106R+6gDNA
DIGDIIRGKDLYLRYNRKDKTDKLQEQLKKYFQKIYKELTWTATKNGQKSAKDHY
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>9106R+14gDNA
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LNRETVWKAITCGHPDGTYFRNTCAGGTTPTPNKCQCIDQTVPTNFDYVPQYLR
>9106R+7gDNA
DIGDIIRGKDLYLRDKGKRDELEKNLKRIFGIIYGKLKNGKTNGQKSAKEYYKDDT
DGNYYKLREDWWDANREKVWKAITCNAGGGKYFRNTCDGGQNPTETQSNCQ
CINLDVPTYFDYVPQYLR
>9106R+15gDNA
DIGDIVRGRDLYRRDKGEETKLEKNLKEIFRRIQGKNQSKLGALSLDKVREYWWE
LNRETVWKAITCGHPDGTYFRNTCAGGTTPTPNKCQCIDQTVPTNFDYVPQYLR
>9106R+8gDNA
DIGDIVRGRDIFRGNDEEKTKRDELESKLKEIFGKIKGNNNSTLTGLPLHKVREYW
WYANRETVWKALTCNAGGYSYFRATCGDSGSPSMARDKCRCKDENLTKETDQ
VPTYFDYVPQYLR

9106R- gDNA sequence

>9106R-2gDNA
DIGDIVRGRDMFKPNSDDKVQEGLKVVFRKINDGLKKSRINDYDRDGPEYYKLRE
DWWTANRNQVWRAITCAARYNPGYFKKSDRSTQVFSNPKCGHDEGIVPTNLDY
VPQFLR
>9106R-10gDNA
DIGDIIRGKDMYVGYDEKEKNRRKQLEDKLKEIFKKIHDDVMKTSGKNVDKAKAR
YNDGSGNYYQLREDWWTLNRKDVWKALTCSAPGDAKYVKYFPSNTTTVSYNQ
CGHNDMNVPTNLDYVPQFLR
>9106R-11gDNa
DIGDIVRGRDLYSGNKKKDKLEENLNKIFNDIKNKNKTKLGELTLDQVREYWWEE
NRQQVWKAITCNATGGKYFRNTCGSGKTASATYDKCHCIGGEVPTYFDYVPQY
LR
>9106R-4gDNA
DIGDIIRGKDLFIGYDQKEKDRRENLENKLKKIFEKIHKEVTSTSGRRNRQTLKARY
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RCTTHDVPTYFDYVPQYLR
>9106R-12gDNA
DIGDIIRGKDLYLRYNRKDKTDKLQEQLKKYFQKIYKELTWTATKNGQKSAKDHY
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RCTTHDVPTNFDYVPQYLR
>9106R-5gDNA
DIGDIIRGRDLYGGGGRGKGKDKLQDNLKKIFEKIHDDVTSGKQNGELKTRYKDD
AKGGHFYQLREDWWTANRHTVWKAITCKADTGNAYFRTTCSDNRGFSQAHDK
CTCNNGDVPTYFDYVPQYLR
>9106R-14gDNA
DIGDIIRGKDLFRGYNQKDRNEKKQLQQRLKEIFKQIHSEVTNGSNGQALKTRYQ
DDNGGNFFKLREDWWYANRQEVWKAITCDVVSGTYFHATCSERNGGCSQANN
KCRCPMTSDGKPNDQVPTYFDYVPQYLR
>9106R-15gDNA

DIGDIIRGKDLFRGYNETDKEQKVALQQHLKEIFEKIYGELKGEAQKQYKDDNGG
NYFQLREDWWALNRRQVWKAITCKADENDRYSANIEHDRTGVSHERCGHEDD
NVPTNLDYVPQYLR
>9106R-1gDNA
DIGDIIRGRDLYGGGGRGKGKDKLQDNLKKIFEKIHDDVTSGKQNGELKTRYKDD
AKGGHFYQLREDWWNNNRKMVWYAMTCGAGTSAQYFRKTCGSGNWTKDKC
RCTTHDVPTNFDYVPQYLR

8211R+ cDNA sequences

>8211R+9
DIGDIVRGRDLYRGNKQEKKKRDELDENLRKIFKQIHEDVTRGRNGVNSRYKDTT
NFYELREDWWTANRETVWKAITCGTHDGDTYFRTTCNDNGTLSDANHKCRCRS
KNAINETDQVPTYFDYVPQYLR
>8211R+2
DIGDIVRGRDLYRGNRKKNQNETERDKLEEKLKDIFKQIHEDVTSTNGKTNGAEA
RYGKDPNFFQLREDWWTANRETVWKAITCNADTGNAYFRTTCSDGKSQSQAN
KYCRCNDDKPGEDKPNIDPPTYFDYVPQYLR
>8211R+10
DIGDIIRGRDIFLGNDEEKKKREDLEKNLKEIFKNIKNENTDLKKLTTEKVREYWWY
ANRATIWEAMTCKAEGAYFHATCSDGRGGAQANKHCRCEGANVVPTYFDYVP
QFLR
>8211R+1
DIGDIVRGKDLFLGNDKEKEKRKQLDDKLKEVFGNIYNELRSTNGVKSRYENDTA
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>8211R+3
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DNQVPTYFDYVPQYLR
>8211R+11
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>8211R+4
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VLTNLDYVPQYLR
>8211R+12
DIGDIIRGKDLFLGNTYESAQRKQLDKNLKRIFKKIYKEVTKTNEELKRRYEGDTK
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CKDKNGSPDDQVPTYFDYVPQYLR
>8211R+5
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RCAAGGVPTYFDYVPQYLR
>8211R+13
DIGDIVRGKDMFKRTDKDKKKEGLDVVFKKITNTLPSPAQNYYAYESENYYKLRD
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YVPQFLR
>8211R+6

DIGDIVRGKDLYGGNNKRRQQLEENLRKKFANIYEELSRTPTKNGQKSAKDRYG
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CTCAAGDVPTYFDYVPQFLR
>8211R+8
DIGDIVRGKDMFKRTDNDEVWKGLRAVFKKIHDNLSSEVKNAYPDDGSGNYYKL
REDWWTANRNQVWRAITCAARYNPGYFKKSDTSTQVFSNPKCGHDEGTVPTN
LDYVPQFLR

8211R- cDNA sequences

>8211R-9
DIGDIIRGKDLYIRNKQEKDRLEENLQKIFANIYEELTNGAQTYYNDDKENYYKLRE
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VPQYLR
>8211R-2
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VXXFLR
>8211R-3
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>8211R-4
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>8211R-12
DIGDIVRGRDLYRGNKQEKKKRDKLENKLKEIFGDIYKELTTSRKKSAEAEARYKD
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>8211R-5
DIGDIVRGKDLFRGYNETDKEHKVALQQHLKEIFKKIHEGLTTTKGAEARYKDDG
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DVPTYFDYVPQYLR
>8211R-6
DIGDIVRGKDLYLGNPQESARRDKLENKLKEIFGDIYKELTTSRKKSAEAEARYKD
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TNDVPTYFDYVPQYLR
>8211R-7
DIGDIVRGKDLFLGNNESEIKRKEKLRGNLXXIFEKIKTSNTNMNTLSLDKVREYW
WALNRKDVWKALTCNAPYEAQYFIKSSDKEHSFSSEYCGHHNNDDPLTNLDYV
PQFLR
>8211R-8
DIGDIVRGKDXFLGNDKEKEKRKQLDDKLKEVFGNIYNELRSTNGVKSRYENDTA
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DNQVPTYFDYVPQYLR
>8211R-11
DIGDIIRGKDLYIRNKQEKXRLXXNLQXIFGXXYEELTNGAQTYYNDDKENYYKLR
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XXXYLR

8211 R+ gDNA sequences

>8211R+9gDNA
DIGDIVRGRDLYRRDKGEETKLEKNLKKIFEKIHSDVTKTSDKNVDKAKERYNDTE
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CRCDGDEPKADKQNVDPPTYFDYVPQYLR
>8211R+2gDNA
DIGDIVRGRDLFHGNDKEKEKRDELDKKLKTIFDKIKKSDEKLTSLKDEQIREYWW
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XYLR
>8211R+10gDNA
DIGDIIRGKDLFLGHQQRKIQLEERLEQMFKNIQKNNANLEGLSIEKVREYWWEA
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>8211R+12gDNA
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YKCRCEGANVVPTYFDYVPQYLR
>8211R+13gDNA
DIGDIVRGKDLYLGNPQESARRDKLENKLKEIFGDIYKELTTSRKKSAEAEARYKD
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TNDVPTYFDYVPQYLR
>8211R+14gDNA
DIGDIVRGKDLFLGNTYESAQRKKLDDKLKGVFAKIHSEVTNGNKDAKDRYKDTK
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QVPTYFDYVPQFLR
>8211R+7gDNA
DIGDIVRGKDLYLGKKKKKKKXTERDXLXQXLKXXFGKIHSEVXXXNGAKXRYXG
DTDNYYELREYXXALNXQTVWKAITCDAPXEAQXFGGTXXGEKIXTLAKYKCRC
DDKPKTGKKPGXVNIVPTYFXYVPQYLR

8211 R- gDNA sequences

>8211R-9gDNA
DIGDIVRGKDLYRGYDDEEKKKRDELDKKLKTIFGKIHSDVTKTSDKNVDKAKERY
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AHHYCRCDGDEPKADKQNVDPPTYFDYVPQYLR
>8211R-2gDNA
DIGDIVRGRDMFKSNNDVEKGLHVVFKKIQRKLNGAAKSYCNADEKGNFYKLRN
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DYVPQFLR
>8211R-10gDNA
DIGDIIRGKDLYSGNSKEKKKRDELXKNLKKIVENIKKENKSKLKSLTDEQIREYW
WELNREMVWYAITCEAPKDSKYFRPTCGSGEWTKDNCRCVKNDVPTYFDYVP
QYLR
>8211R-11gDNA
DIGDIIRGKDLYSGNSKEKKKRDELEKNLKKIFENIKKENKSKLKSLTDEQIREYW
WELNREDVWKAITCDVVSGKYFRNTCGSGEKGSATYEKCRCNDDQVPTYFDYV
PQYLR
>8211R-4gDNA
DIGDIVRGRDLFHGNDKEKEKRDELDKKLKTIFDKIKKSDEKLTSLKDEQIREYWW
ALNRQDVWKAITCDVVSGKYFRNTCGSGEKGSATYEKCRCNDDQVPTYFDYVP
QYLR
>8211R-12gDNA

DIGDIVRGKDLFYGNPQEKEQREKLDEKLKTIFGKIHEGLDPKIKSNYNDAPYYYK
LREDWWNANRETVWKAITCDVKSGNNYFRQTCGDEKTGTLTPNQCRCDGDQV
PTYFDYVPQYLR
>8211R-5gDNA
DIGDIVRGKDLYRGYDDEEKKKRDELDKKLKTIFGKIHGGLTDQKAKQHYNGDTK
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RCAAGGVPTYFDYVPQYLR
>8211R-6gDNA
DIGDIVRGKDLFRGNDEEKEKRKQLEKNLKKIFGNIYKELKKKKKGRNGQIEARYK
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>8211R-7gDNA
DIGDIVRGRDLYGGNNKRRQQLDKKLKEIFKNIYDNLMEDLKKYPTKKVEAEKHY
QDKNGGNFFKLREDWWTANRHTVWEAITCDNKLAGASYFRATCGDSESPSQA
QNQCRCPKSRDNKPNDQVPTYFDYVPQFLR
>8211R-15gDNA
DIGDIIRGKDLYSGNSKEKKKRDELEKNLKKIFENIKKENKSKLKSLTDEQIREYW
WELNREMVWYAITCEAPKDSKYFRPTCGSGEWTKDNCRCVKNDVPTYFDYVP
QYLR

9197R+ cDNA sequences

>9197R+4
DIGDIVRGKDPFYGNTQESARREDLEKNLKEIFGNIYEELIKNGRNGVKDHYNDA
KKNFYRLREDWWTANRATVWEALTCEVGSGTYFRPTCSDSGDRQGPSVAQKQ
CRCPNGNNQVPTYFDYVPQYLR
>9197R+14
DIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYWWA
LNRVQVWKAITCDATMNDIFSKNIXNSXTTLFYYKCGHYVYKDVPTNLDYVPQYL
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>9197R+1
DIGDIXRGKDLFRGNKQESAQRIILENNLKTIFKKIHDDVTNRKTNKEAAEARYKDE
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NVLTNFDYVPQYLR
>9197R+6
DIGDIVRGKDLFRGNKQESAQRIILENNLKXIFKKIHDDVTNRKTNKEAAEARYKD
ESGNYYQLREDWWDANRATVWEAITCEAPKDSKYFRGTCSTGTATYEKCRCAS
GNVLTNFDYVPQYLR
>9197R+13
DIGDIVRGKDLFRGNKQESAQRIILENNLKTIFKKIHDDVTNRKTNKEAAEARYKDE
SGNYYQLREDWWDANRATVWEAITCEAPKDSKYFRGTCSTGTATYEKCRCASG
NVLTNFDYVPQYLR
>9197R+11
DIGDIVRGKDLFRGNKQESAQRIILENNLKTIFKKIHDDVTNRKTNKEAAEARYKDE
SGNYYQLREDWWDANRATVWEAITCEAPKDSKYFRGTCSTGTATYEKCRCASG
NVLTNFDYVPQYLR
>9197R+2
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GDKNNNFFKLREDWWYANRRQVWKAITCDAKAFNYFRNTCNGESPTKGYCRC
NDDKPDDDKPNTDPPTYFDYVPQFLR
>9197R+5

DIGDIVRGRDLYSGNKKENKQRKQLEDNLKKIFENIKNENTELSTLTTEKVREYW
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KADQVPTYFDYVPQFLR
>9197R+9
DIGDIIRGKDLFYGNPQESAQRKVLDEKLKTIFKQIHDDVMKTSSNNKEVLKTRYK
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RCPSYKVPTYFDYVPQYLR
>9197R+7
DIGDIVRGRDMFKSNEKVEIGLXKVFXKINNGLXKIGIHDXXDIXGNYYXXXDXWW
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FLR

9197R- cDNA sequences

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RCPSYKVPTYFDYVPQYLR
>9197R-4
DIGDIVRGRDLFYGNTQESARRDKLEENLKRIFGKIYEELTKDKKKTVEAKKKYKD
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HNEENVLTNLDYVPQFLR
>9197R-14
DIGDIVRGRDLFYGNTQESARRDKLEENLKRIFGKIYEELTKDKKKMVEAKKKYKD
ENGGNFFKLREDWWTANRETVWKAITCAAKVGDTYFMESRTNSYKFSNGQCG
HNEENVLTNLDYVPQFLR
>9197R-10
DIGDIVRGKDLFRGNKQESARIILENNLKTIFKKIHDDVTNRKTNKEAAEARYKDGS
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NVLTNFDYVPQYLR
>9197R-2
DIGDIVRGKDLFRGNKQESAQRIILENNLKTIFKKIHDDVTNRKTNKEAAEARYKDE
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NVLTNFDYVPQYLR
>9197R-6
DIGDIVRGRDLFRGNDEEKKKREDLENKLKEIFGKIHKEVTSGRTNGEIEARYKGD
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VLTNFDYVPQYLR
>9197R-5
DIGDIVRGRDLYGGGGRRKAELQDNLKTIFGKIHSGLTSGVKARYEGDKENYYQL
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KPSENNPNTDPPTYFDYVPQYLR
>8148R-6
DIGDIVRGKDLYGGGGRGKGKEKLXKXXKEIXXKIHEKLDSKAQTYYNDDKENYS
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PGKDKANTDPPTYFDYVPQYLR
>9197R-9
DIGDIIRGRDLYRGNKKKKLNGKETERDQLENKLKTIFGNIYEELTTSREKSAKAK
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ATQGNCRCVIGGVPTYFDYVPQFLR
>9197R-12

DIGDIIRGKDLYIGNRKEKEKVKLQNNLKGIFKKIYGELKNTKAKVHYQEDGPNYY
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>9197R-11
DIGDIIRGKDLFYGNPQESAQRKVLDEKLKTIFKQIHDDVMKTSSNNKEVLKTRYK
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RCPSYKVPTYFDYVPQYLR
>9197R-15
DIGDIVRGKDLFRGNKQESAQRIILENNLKTIFKKIHDDVTNRKTNKEAEAGYQNE
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RCASGNVLTNFDYVPQYLR

9197R+ gDNA sequences

>9197R+6gDNA
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R
>9197R+3gDNA
DIGDIVRGRDLYRGNKKKKLNGKETERDQLXNKLKTIFGNIYEELTTSREKSAKAK
ERYKKDEDGNYSKLXEDWWNNNRKMVWIAMTCEAGGDKYFRNTCGSDENHPT
ATQGNCRCVIGGVPTYFDYVPQFLR
>9197R+13gDNA
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IGDVPTYFDYVPQFLR
>9197R+1gDNA
DIGDIIRGKDLYLRNKGEKKKRDELEKNLKKIFGIIYNNLFKKKGEEAQKRYNDEDK
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PTYFDYVPQYLR
>9197R+2 gDNA
DIGDIIRGKDLYLRNKGEKKKRDELEKNLKKIFGIIYNNLFKKKGEEAQKRYNDEDK
NFFKLREDWWTANRHTVWKALTCDARDNADDKYFRKTACGEGTTSTQGKCRC
PNGDQVPTYFDYVPQYLR
>9197R+10gDNA
DIGDIVRGKDLYLGDKKEKLDLEKKLKDIFAKIYGELKDAKEHYKDESRNYFQLRE
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TYFDYVPQYLR
>9197R+11gDNA
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>9197R+9gDNA
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RCAAGDVPTYFDYVPQFLR
>9197R+4gDNA
DIGDIVRGKDPFYGNTQESARREDLEKNLKEIFGNIYEELIKNGRNGVKDHYNDA
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CRCPNGNNQVPTYFDYVPQYLR

9197R- gDNA sequences

>9197R-3gDNA
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>9197R-1gDNA.
DIGDIVRGKDLYRRDNKKDKLEXNLKRIFXXIYDKLKNGKXKGKEAKDRYGKDPN
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>9197R-13gDNA
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DYVPQYLR
>9197R-12gDNA
DIGDIVRGRDLFRGNDEEKKKREDLENKLKEIFGKIHKEVTSGSNKDALKTRYNG
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DDKKPDKSKAGNGDVNIVPTYFDYVPQFLR
>9197R-10gDNA
DIGDIVRGKDPFYGNTQESARREDLEKNLKEIFGNIYEELIKNGRNGVKDHYNDA
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8148R+ cDNA sequences

>8148R+10
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>8148R+12
DIGDIVRGRDLYLGGRGRDQLESNLKKIFGIIYNNLMQDLTNDQTKSAEAEARYK
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CINFSVPTYFDYVPQYLR
>8148R+15
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PTYFDYVPQFLR
>8148R+7
DIGDIVRGRDLYRGDKGEKKYLEENLKQMFENIKGNNNKLGRLSTKQVREYWWA
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LR
>8148R+9
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>8148R+16

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PQFLR
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XYVPQFLR
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>8148R+6
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>8148R+4
DIGDIVRGKDLYFGKKKKKQTERDQLESKLKEIFGDIYNELTTTNGVKERYKDDRE
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8148R- cDNA sequences

>8148R-4
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>8148R-14
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>8148R-11
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>8148R-10
DIGDIVRGKDLFLGNDKEKKQRDKLENNLKKIFEDIYEELKKDRNLKEGAQKRYG
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>8148R-13
DIGDIVRGRDPFYGNTXEKEKXEQLEXNLKXXFGKIYEKLNGAKERYNDNDENYY
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XXGKPXSNAGKGSGNGDVNIVPTYFDYVPXFLR
>8148R-5

DIGDIVRGRDLYGGNNKEKKQRQQLENNLKEIFENINKSDTKLSTLEDDQIREYW
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YFDYVPQFLR

8148R+ gDNA sequences

>8148R+1gDNA
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>8148R+2gDNA
DIGDIIRGKDLYLGNGDYKVKLSNNLREIFKNIYDALEDTVKENYKDTTNYYKLREY
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PQYLR
>8148R+14gDNA
DIGDIXRGRDMFKRTDKDAVQKGLRAVFKKIYDNLSSSVKQHYKEDKDENYYKL
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LDYVPQYLR
>8148R+9gDNA
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TCNNGDVPTYFDYVPQFLR
>8148R+12gDNA
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>8148R+10gDNA
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>8148R+3gDNA
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8148R- gDNA sequences

>8148R-2 gDNA
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>8148R-11gDNA
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AANIDPPTYFDYVPQYLR
>8148R-13gDNA
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TYFDYVPQYLR

>8148R-14gDNA
DIGDIXRGKDLYLGNKGEKKKRDELEKNLKKIFKEIHGGLTKANGVKDHYQDDGP
NYYKLREDWWTVNRHTVWKAITCNEDKKLKDASYFRKTCSDRQGESVAIHYCR
CNGDKPDNDMVNIDPPTYFDYVPQYLR
>8148R-4gDNA
DIGDIVRGRDLYRGDKGEKKYLEENLKQMFENIKGNNNKLGRLSTKQVREYWWA
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LR
>8148R-10gDNA
DIGDIIRGRDLYFGNNKEKKQRQQLDDKLKEIFGKIHEGLTDSGAKNHYNDPSGN
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8.5 Full-length SA075var1 nucleotide sequence

>SA075var1
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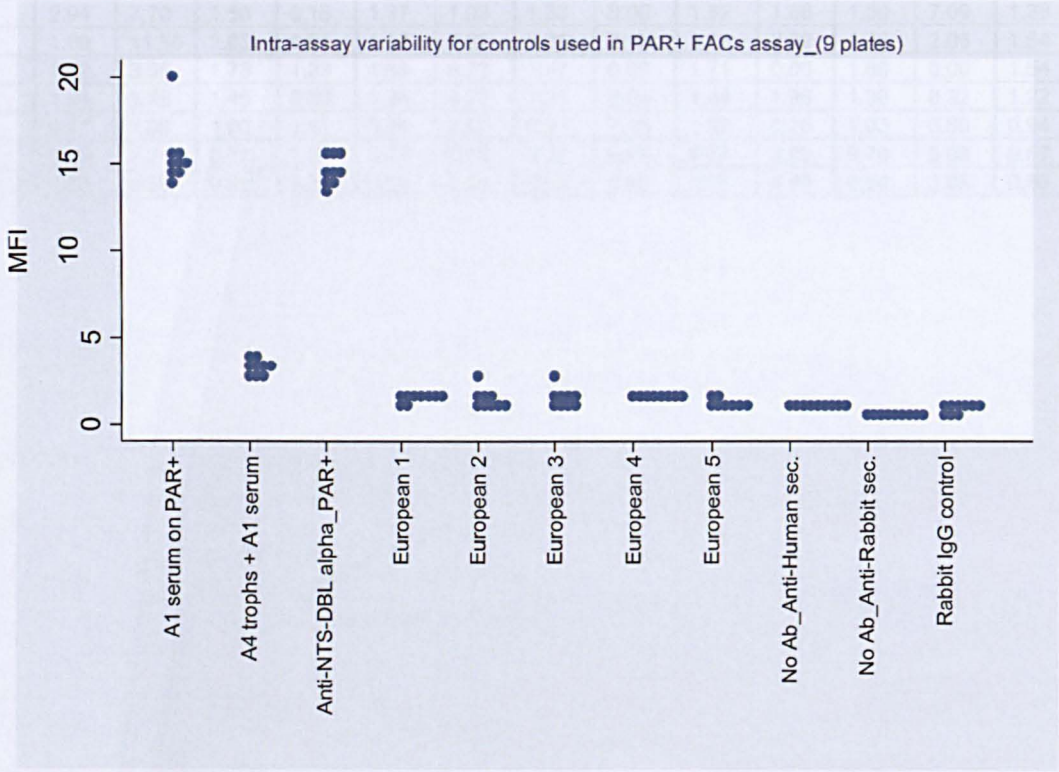
8.6 : Assay variability for population based study

Graphs showing intra-assay variability across 9 plates



% CV FOR DUPLICATE READS ACROSS 9PLATES _PAR+

	Plate 1		Plate 2		Plate 3		Plate 4		Plate 5		Plate 6		Plate 7		Plate 8		Plate 9	
Sample	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV
A1 serum on PAR+	14.80	17.20	15.75	3.14	20.15	11.58	14.85	39.52	14.30	6.92	14.95	4.26	15.80	10.74	14.20	8.96	14.05	7.55
A4 trophs + A1 serum	2.66	16.48	2.68	10.84	2.94	0.72	3.27	9.31	3.94	9.52	3.53	14.64	4.04	5.78	3.27	1.73	3.21	15.22
Anti-NTS-DBL alpha_PAR+	14.40	3.93	14.30	3.96	15.55	1.36	15.55	0.45	14.55	0.49	13.70	8.26	15.35	6.91	14.05	2.52	13.35	3.71
European 1	1.88	24.82	1.50	3.31	1.74	6.93	1.71	2.07	1.74	4.06	1.41	3.52	1.77	22.37	0.98	26.66	1.34	7.39
European 2	2.94	7.70	1.50	6.15	1.37	1.03	1.33	8.00	1.52	1.86	1.50	7.09	1.38	4.10	1.20	4.71	1.26	3.94
European 3	1.59	11.56	2.65	6.91	1.47	0.96	1.35	2.10	1.62	2.62	1.38	2.05	1.54	1.38	1.28	5.52	1.26	0.56
European 4	1.62	3.06	1.73	1.23	1.58	6.73	1.47	0.96	1.71	0.00	1.68	0.00	1.54	0.00	1.46	11.18	ND	ND
European 5	1.35	5.78	1.45	0.00	1.34	4.22	1.23	8.05	1.44	1.96	1.36	8.32	1.22	15.71	1.25	7.38	ND	ND
No Ab_Anti-Human sec.	0.97	7.60	1.00	1.41	0.94	2.62	0.91	7.15	1.02	0.70	1.03	0.69	0.94	1.59	0.92	1.53	0.91	3.59
No Ab_Anti-Rabbit sec.	0.70	2.34	0.71	1.29	0.77	0.18	0.72	9.10	0.77	3.93	0.76	3.08	0.67	5.49	0.69	2.36	0.70	4.25
Rabbit IgG control	0.83	0.59	0.89	1.35	0.92	0.08	0.94	0.45	0.93	4.49	0.94	3.08	0.85	3.48	0.77	4.52	0.76	3.06

Intra-assay variability for controls used in SAO75R+ FACS assay (9 plates)

% CV FOR DUPLICATE READS ACROSS 9PLATES _SAO75R+

	Plate 1		Plate 2		Plate 3		Plate 4		Plate 5		Plate 6		Plate 7		Plate 8		Plate 9	
Sample	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV	Mean	% CV
A1 serum on ASO75R+	30.86	10.72	37.95	21.97	23.66	0.66	27.16	7.99	26.29	4.20	22.59	1.19	25.23	1.29	26.20	1.03	25.09	0.06
A4 trophs + A1 serum	2.72	1.04	2.98	3.57	1.96	6.49	1.82	24.09	1.92	21.05	1.83	13.91	2.03	10.45	2.16	1.31	2.48	14.83
European 1	0.06	12.86	0.14	1.02	0.15	2.41	0.01	9.43	0.02	35.91	0.12	17.39	0.09	19.11	0.10	7.56	0.21	10.13
European 2	0.04	25.36	0.15	6.83	0.15	4.12	0.01	23.57	0.09	8.14	0.13	33.83	0.08	3.54	0.04	15.71	0.08	9.96
European 3	0.14	22.48	0.14	3.03	0.14	3.03	0.01	17.85	0.09	28.95	0.09	16.97	0.11	4.56	0.04	13.56	0.09	3.14
European 4	0.13	13.65	0.22	0.94	0.22	10.83	0.13	9.05	0.19	4.75	0.01	121.22	0.14	10.80	0.10	8.66	ND	ND
European 5	0.12	7.31	0.18	5.88	0.18	2.00	0.03	16.79	0.11	3.29	0.03	14.63	0.12	13.84	0.12	30.18	ND	ND
Anti-NTS-DBL alphaSAO75R+	15.38	14.88	15.94	1.00	14.14	2.54	13.40	1.45	14.25	0.73	14.51	1.94	14.63	2.79	14.11	2.02	14.56	6.28
No Ab_Anti-Human sec.	0.11	1.07	0.16	3.03	0.31	18.11	0.10	5.08	0.03	6.53	0.03	62.23	0.07	11.52	0.06	8.30	0.10	2.40
No Ab_Anti-Rabbit sec.	0.13	10.22	0.24	26.32	0.26	28.50	0.11	12.86	0.02	141.42	0.03	28.28	0.13	2.79	0.04	24.51	0.16	22.92
Rabbit IgG control	0.28	15.54	0.34	10.15	0.37	1.74	0.08	37.49	0.18	1.21	0.11	19.31	0.24	7.32	0.20	18.57	0.25	8.59

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